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Editor
HAMILTON B. G. ROBINSON

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DECEMBER, 1955

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EDITOR'S VIEWPOINT

Kreshover (p. 798) applied tobacco smoke to the skin of mice and confirmed his earlier observation of increased susceptibility to irritation in vitamin B complex deficient animals. Riboflavin, pyridoxine and pantothenic acid appeared to be primarily responsible. Gonadectomy reduced susceptibility to tobacco irritation. Heat was ruled out as a probable cause of tissue change in these animals for the temperature rise on the tissues was only 1.8° Centigrade.

Some very interesting structures may be observed from electron microscopic studies of dentin by replicas. Yasuzumi and Obata (p. 808) show a nonfibrous membrane about Tomes' fibers, the structure which they interpret as the controversial sheath of Neumann surrounded by collagenous fibers. Uncalcified collagen fibers appeared in the dentinal matrix and tubules. These and other studies by electron microscopy are still not fully interpreted and one must be cautious in drawing conclusions until the photographs are compared with studies made chemically, by x-ray diffraction and by polarized light, among other technics.

Spectrographic analysis gives information on elements in "trace" amounts. Using hamster teeth, Burnett and Lobene (p. 814) searched for 34 possible trace elements. Of these, 17 were found consistently in enamel with two others noted on occasion. Dentin showed 16 elements regularly and 3 occasionally. Of course, calcium and phosphorus were the major elements with manganese, sodium and iron also above 0.1 per cent. Studies of trace elements may lead to information on variations in caries susceptibility (or acid solubility) and to better means of controlling dental caries.

Hartles and Leaver (p. 820), investigating the fluorescing fractions of sperm whale teeth, identified thymine in cementum and, to a lesser degree, in dentin. An ether soluble basic pyrimidine was also found in dentin. They suggest that these pyrimidines may be derived from nucleoproteins and that odontoblastic processes may be involved. Studies of the organic fractions of hard dental tissues have been sparse and this approach, using larger teeth, may help to supply important information.

Photoelasticity has been applied in industry and in science to measure stresses. Mahler and Peyton (p. 831) discuss the principles involved and the application of photoelasticity in dentistry. Since preparation of their paper Haskins, Haack and Ireland have published a photoelasticity study of dental restorations (J. D. Res. 33: 757, 1954).

Phillips, Phillips, and Schnell (p. 839) summarize their study of electric conductivity of dental cements, and conclude that cement bases, cavity liners, and silver nitrate fail to inhibit conduction of electricity through the tooth. The tooth itself is a good conductor. Pain resulting from electrical currents produced by dissimilar metals ceases when the tooth protects itself by sclerosis of dentin, secondary dentin or other means.

Following a clue from industry, Buoncore (p. 849) used phosphoric acid and phosphomolybdate-oxalic acid on enamel surfaces, in vivo, in an attempt to increase adhesion of acrylics. The former increased adhesion from an average of 11 hours to 1,070 hours. While not resulting in positive adhesion the use of phosphoric acid is suggested to improve sealing of acrylics in pits and fissures.

Silberkweit, Massler, Schour and Weinmann (p. 854) utilizing the growing incisor of the albino rat as a tool for testing, state the effects of various filling materials on the dental pulp. Their findings are comparable with those obtained on human teeth and teeth of other animals. Zinc-oxide and eugenol appears to minimize injury to the pulp while silver amalgam, self-curing acrylic, silicates and copper cements led to pulp damage. A calcium hydroxide liner gave evidence of some protection against injury by silicate cements. This observation is worthy of more intense investigation

as some clinicians have been using this material as a cavity liner on an empirical basis.

Based on casual observations, an association between socio-economic level and dental caries has often been claimed. Savara and Suher (p. 870), from careful parent interviews of 279 pre-school children, found no relationship between parents' education or parents' income and dental caries. Parents' income did influence the number of times the children brushed their teeth daily. A further study of this type on older children may reveal interesting trends and suggest revisions in some of our concepts of home care.

Studies in implantation of lactobacilli into the oral cavity are especially difficult because of the universal appearance of lactobacilli in mouths which permit their growth. Using hamsters on cariogenic and regular diets Palmer, Laffer, and Faber (p. 876) attempted to implant a laboratory strain of *L. acidophilus.* Since all of the animals demonstrated salivary lactobacilli, in reality this was an attempt to increase the numbers of lactobacilli. The counts in the animals on regular diets were significantly different for a short period only. The salivary lactobacilli counts were closely related to caries activity but there was no absolute correlation with numbers of carious lesions and gross loss of tooth structure. The hamster, a coprophagic animal, is a difficult subject for such studies. The oral flora of these, and other laboratory animals, deserves more thorough investigation.

Rae, Shemilt, and Clegg (p. 886) found that the optimal range for ammonia production in saliva is pH 6.5 to 7.0 but that the inhibitory effect of added glucose in saliva is not due to lowering of the pH alone. They also found that the stimulation by glucose of ammonia production in saliva-urea mixtures may be the result of the glucose keeping the pH near optimum levels.

An interesting, and still unexplained, group of studies on rats (p. 889) has led to the finding that orchiectomy at 30 days of age results in significant reduction in their dental caries and ovariectomy at 30 days only tends to reduce caries. At 60 days neither operation affects caries activity significantly. That these effects are not due to salivary gland reactions alone seems to be shown by further caries reduction by castration in desalivated animals. These results of Bixler, Muhler, and Shafer may become fully significant when more is known of the reason for the castration-caries effects.

Muhler and Weddle (p. 895) found that as the pH of fluoridated drinking water given to rats is increased from 2 to 4 the effectiveness of stannous fluoride and stannous chlorofluoride in reducing dental caries is lowered. Of course, such pH levels for fluoridated waters used for community consumption do not seem practical, the project being part of a long-range program of fluoride studies and not directed at recommendations for public health use. Even at higher pH levels stannous chlorofluoride seemed more effective than other fluorides tested.

Weddle and Muhler (p. 900) found that rats fed a given quantity of fluorine in water once per day stored more fluorine in the skeleton than those fed an equal amount daily but divided into 3 doses. Whether frequency of intake of fluoridated water would result in any significant difference in protection from dental caries cannot even be accurately conjectured from these data but the value of a study of the effect of frequency of intake on caries rates is apparent.

Desalivated rats show histologic changes in the adrenal glands more pronounced in the male than in the female. Bixler, Muhler, and Shafer (p. 910) suggest that this may be the result of reduced food intake and therefore of nutritional nature. The possibility of desalivation affecting the general adaptation of the animals should also be considered.

Sreebny, Meyer, and Bachem (p. 915) studied the enzyme system associated with proteolytic activity of the submaxillary gland of the rat. The system appeared to be nonspecific. Investigations of enzymatic activity in whole saliva, as removed from the mouth, are difficult to interpret because of the possible roles of salivary gland secretion of tissues lining the oral cavity, and of microorganisms of the oral flora. The direct approach to the problem by measuring activity in tissue of the gland is less oblique and leaves less room for controversy.

Flieder and Fisher (p. 921) used the Warburg method for determining the oxygen consumption of dental pulps from young cattle. The oxygen consumption they observed was higher than had been found for human pulps and lower than for most other tissues. It is interesting that in removing the pulps the odontoblasts were left behind. The odontoblasts are more intimately associated with the dentin than they are with the pulp. Judging from their histologic appearance and life history, one would not expect the odontoblasts to markedly increase oxygen consumption in the pulp.

Schultz-Haudt and Scherp (p. 924) isolated streptococci from gingival crevices of patients with marginal gingivitis and from individuals with healthy gingivae. Hyaluronidase was produced by almost one-half of the strains from gingivitis patients and by about one-twentieth of the strains from gingivitis-free individuals. Beta-glucuronidase was also produced more frequently by streptococci from diseased crevices. Although numbers and species of bacteria in gingival crevices have not been correlated with gingivitis, the authors propose that their enzyme production may be. On this basis, the organisms involved in gingival disease would be quite specific on the basis of their clinical activities but not their species.

H. B. G. R.

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FURTHER OBSERVATIONS ON THE EFFECT OF TOBACCO ON EPITHELIAL TISSUES OF VITAMIN-DEFICIENT MICE

SEYMOUR J. KRESHOVER

The Medical College of Virginia, School of Dentistry, Richmond, Va.

A PREVIOUS study related to the effect of tobacco on epithelial tissues of mice indicated the uncertainty and questionable validity of making clinical application from observations on laboratory animals.[1] Similar difficulties are apparent in the inconclusive nature of clinical studies based largely upon statistical methods. However, a growing concern about the possible role of tobacco as an etiological factor in cancer would seem to warrant appreciably more study in both categories of research to the end of resolving many of the apparent inconsistencies that have developed in the literature.

It has been the primary purpose of the present series of studies to evaluate the comparative effects of tobacco on cutaneous and oral mucosal tissues. Additional objectives were to explore further the influence of various systemic and local factors on tissue susceptibility. In this regard, it is of interest to note that, although some reports have indicated a direct relationship between cancer of the lung and exposure to tobacco smoke,[2-4] the incidence of oral leukoplakia and cancer has not appeared to show a corresponding significant rise associated with the increased use of tobacco in recent years.[5]

In the preliminary phase of the present study, it was reported that whole tobacco smoke applications to the lips of mice caused little or no tissue change. These animals had been grouped previously so that one series was maintained on a normal adequate diet, another was rendered deficient in vitamin A, and a third deficient in the vitamin B complex. Two additional groups were comprised of gonadectomized mice maintained, respectively, on normal and B complex–deficient diets. In striking contrast to the negative lip findings in all of these groups was the evidence of appreciable susceptibility of the ears of certain of the same animals following identical procedures of tobacco smoke application. These changes were most marked in the vitamin B–deficient mice, less so in the vitamin A–deficient and normal diet groups, and least in the gonadectomized animals regardless of their diets. On the basis of the observed difference in susceptibility of oral and cutaneous tissues, it was suggested that a significant protective effect is exerted by the tongue and saliva in readily removing deposited tars from the oral mucosa. It was further concluded that, whereas vitamin B complex deficiency causes a markedly increased susceptibility of cutaneous tissues to tobacco irritants, orchiectomy and ovariectomy establish

This study was supported in part by the Research and Development Division, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-506.

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a certain degree of resistance. Further references to the literature on tobacco and vitamin deficiency factors as related to cancer and leukoplakia were reviewed in this initial report.

Following the previously described preliminary study, it became of interest to investigate more thoroughly the various factors that seemingly contribute or predispose to altered tissue susceptibility. The first of these, presented in the following report, is concerned with the subject of vitamin B deficiency.

MATERIALS AND METHODS

A total of 201 Swiss strain mice between the ages of 10 and 12 weeks were used in this study. On the basis of deficiency states produced prior to tobacco smoke application and maintained throughout the course of the experiment, the following animal groups were established: B complex (37); thiamine (22); riboflavin (23); pyridoxine (23); pantothenic acid (24); niacin (23); and

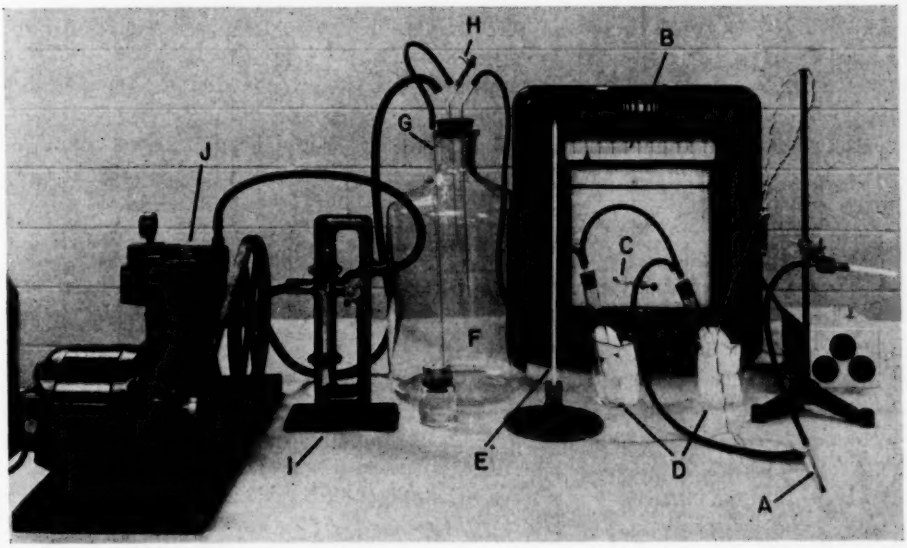

Fig. 1.—Smoking apparatus. Applicator containing thermocouple (*A*); pyrometer (*B*); pinch valve (*C*); smoke traps (*D*); thermometer (*E*); reservoir (*F*); vacuum gauge (*G*); needle valve (*H*); manostat (*I*); vacuum pump (*J*).

biotin (18). Another 6 riboflavin-deficient mice were gonadectomized before receiving like treatment. Of the remaining 25 animals, all maintained on a normal adequate diet, 11 received smoke applications identical to those given the deficient mice, and 14 served as controls in order to evaluate the possible effect on tissues of such factors as pressure and suction incident to the operation of the smoking machine. Each of the established animal groups was comprised of an approximately equal number of males and females.

By means of an apparatus specifically designed for this study (Fig. 1), applications of whole tobacco smoke were made to the posterosuperior region of the right ear of each experimental animal. In all instances, the procedure was

standardized so that the mice received, on alternate days, a like number of repeated applications of equal volume, exposure time, and interval periods. A smoke flow of 17.5 c.c. per second was selected arbitrarily on the basis of an equivalent oral inhalation in the average male smoker. Briefly described, the technique was to apply the applicator tip gently to the selected tissue site and, by manipulating the pinch valve, direct a stream of smoke against the ear for a 5-second exposure period. The circuit then was immediately cleared. This procedure was repeated for a successive series of 5 exposures on each day that an application was made, one cigarette sufficing for each such series. In the case of the control animals, an identical procedure was followed except for using an unignited cigarette. This served the purpose of determining the effect, if any, of suction and pressure alone. Another important factor that was evaluated was the amount of heat created at the tissue site of smoke application. This was determined by means of a thermocouple and pyrometer adapted to the apparatus (Fig. 1).

In addition to daily observations and routine biopsy examinations at varying intervals during the 80-day period of tobacco smoke applications, many of the animals in each experimental group were followed for a further period of 5 months. This procedure was adopted in order to determine whether any observed cellular change of a proliferative nature would be dependent upon repeated exposures to tobacco or to an ultimately acquired intrinsic capacity for progressive growth.

FINDINGS

Inasmuch as the objectives of the present study were to evaluate the effects of whole tobacco smoke per se on certain tissues, it was essential that other possible irritating factors incident to the operation of the smoking apparatus be themselves evaluated. In this manner a more accurate interpretation could be made of any findings related to the various categories of experimental grouping. As noted in Fig. 1, an essential mechanism for operation of the apparatus is a vacuum pump. Although the negative pressure at the site of application is established at a relatively low figure by manostat control, it is conceivable that some tissue irritation might result. A further possible cause of tissue damage might be pressure incident to the repeated contact of applicator tip with skin. Both factors were evaluated carefully by subjecting the ears of 14 mice to the same treatment as that given the experimental animals except for using an unignited cigarette. Following a period of observation equivalent to the maximum number of tobacco smoke exposures, biopsies were obtained for histologic study. No abnormalities were noted (Fig. 2). Grossly, the ears show a smooth, intact surface with no evidence of thickening or induration. The histologic preparations are similarly normal in appearance with a stratified squamous epithelium, arranged in a thickness of 2 to 3 cell layers, covering both surfaces. The rather scanty underlying connective tissue is divided by a thin cartilaginous plate and contains occasional islands of sebaceous cells and hair follicles.

One other possible cause of tissue irritation, unrelated to the effects of tobacco smoke per se, is the factor of heat created at the site of smoke application. On the basis of an average temperature rise of but 1.8° C. when smoke

is ejected onto the tissue, it appears quite unlikely that a pathologic change would result. Such a minor degree of difference between "normal" body surface temperature and that created at a specific site exposed to tobacco smoke is not surprising in light of the relatively long distance of smoke travel from its burning source.

In striking contrast to the absence of pathology in the suction-pressure animals are the rather marked abnormalities resulting from exposures to whole smoke. These varied significantly in degree according to experimental grouping and duration of smoke applications.

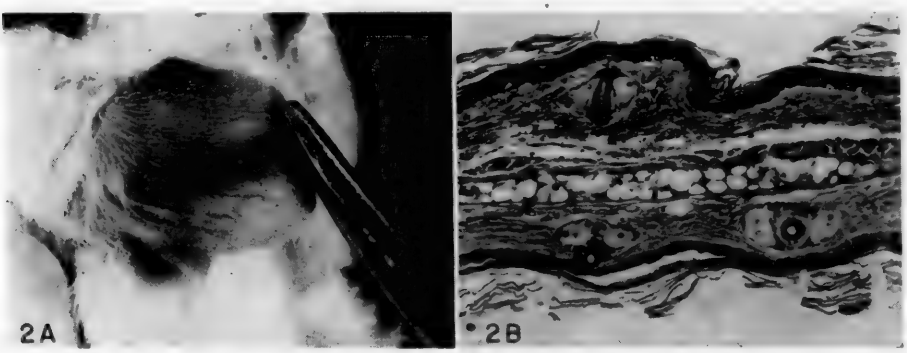

Fig. 2.—*A*, Ear of mouse maintained on a normal adequate diet; 40 days of repeated applications of suction and pressure. No abnormalities noted.

B, Histologic structure of same ear showing normal-appearing epidermis, underlying corium, and cartilaginous plate.

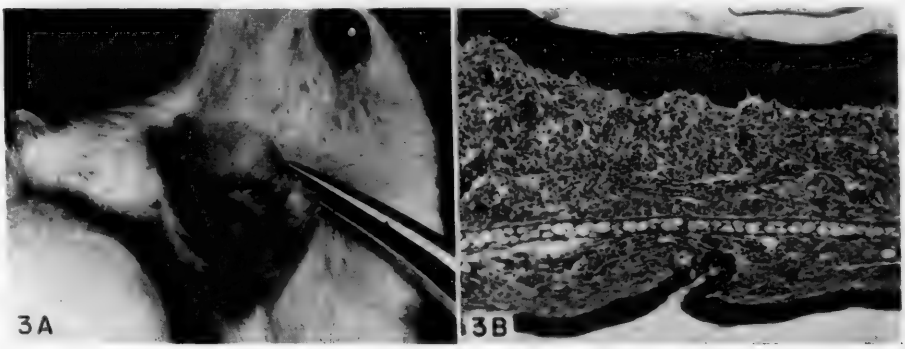

Fig. 3.—*A*, Ear of mouse maintained on a normal adequate diet; 30 smoke applications. The surface has a parchmentlike appearance, and there is slight thickening without induration. Animals treated for longer periods of time show no progression of gross or microscopic change beyond that represented in this or in *B*.

B, Smoked side of ear shows epithelial hyperplasia and a chronic inflammation of the corium with some edema and increased vascularity. The untreated side of the ear is of normal appearance.

Eleven mice were maintained on a normal adequate diet throughout the course of the experiment. The maximal ear changes in this group consist of a slight degree of thickening and an alteration in color and texture which impart a yellow-brown, parchmentlike appearance to the tissue (Fig. 3*A*). Histologically (Fig. 3*B*), the epidermis on the smoked side of the ear shows hyperplasia

as well as some keratinization. The basal-cell region is distinct, uniformly arranged, and well demarcated from underlying corium. Contributing to a moderate thickening of the latter tissue is a round-cell infiltration with edema, increased vascularity, and fibrosis. It is of interest to note that this represents the most advanced type change in the normal diet group and, although oftentimes manifested by the fiftieth experimental day (25 smoke applications), shows no evidence of progression in the postexperimental period of observation. Rather, there is usually a regression to normal-appearing tissue.

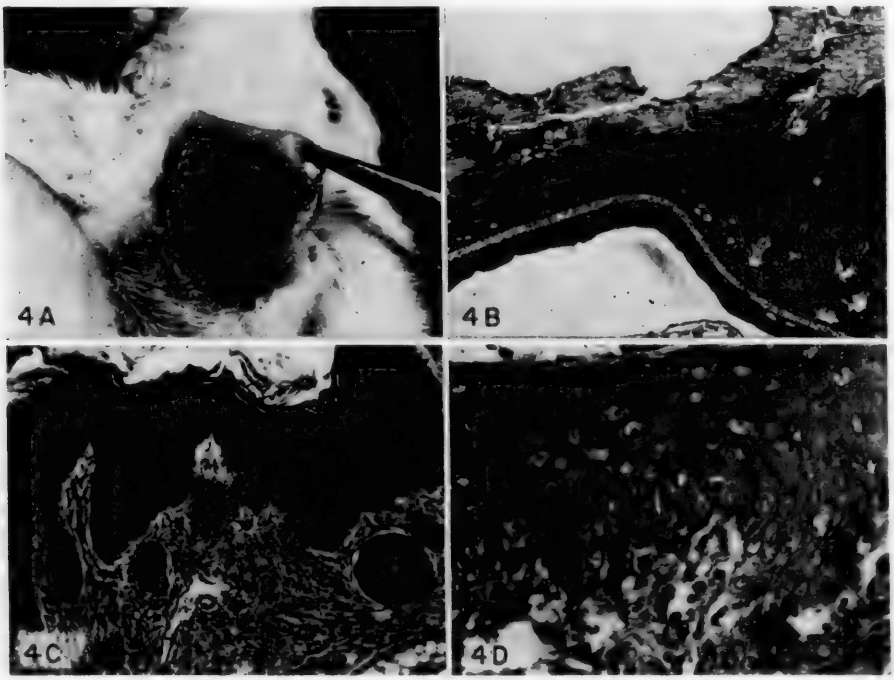

Fig. 4.—*A*, Ear of mouse on vitamin B complex–deficient diet; 38 smoke applications. There is ulceration with scab formation, marked induration, and a raised, rolled border to the lesion.

B, Low-power magnification showing area of ulceration and bordering zone of epithelial hyperplasia. Note the acanthosis and markedly thickened corium. Overlying the entire surface is a thick deposit of tobacco tar and serous exudate. The untreated side of the ear, underlying the cartilaginous plate, shows no abnormalities.

C, Higher magnification of hyperplastic area showing keratinized surface and chronic inflammation of corium.

D, Basal-cell region showing abnormalities in cellular size, shape, and staining qualities. Note the loss of polarity and poorly defined juction between epithelium and corium.

As indicated in a previous report, mice rendered deficient in the vitamin B complex exhibit a markedly greater tissue susceptibility to whole tobacco smoke than do normal diet animals. As demonstrated in Fig. 4, the ears of vitamin B complex–deficient animals are markedly affected by whole tobacco smoke. These changes, consisting principally of ulceration, excoriation, induration, and thickening, often become manifested as early as the fortieth day of smoke application (20 exposures). The thickening is most pronounced in

the area bordering ulceration, thereby contributing a rolled and raised margin to the lesion. It should be noted that the 5-month postexperimental period shows no evidence of intrinsic capacity of the tissue for progressive breakdown or growth. Although healing is apparently of minimal degree, a definite acceleration of repair can be induced by substituting a normal, adequate diet for the deficient one. Microscopically, the areas described show a marked epidermal hyperplasia in regions adjacent to ulceration, as well as a fibroblastic proliferation and hyperemia of the corium (Fig. 4*B*). Overlying such areas is a thick deposit of amorphous material comprised of tobacco tar and serous exudate. In marked contrast to these abnormalities is the relatively unaffected tissue on the inferior or unsmoked side of the ear. Here, sharply demarcated by the cartilaginous plate, is a thin, uniform, and relatively normal-appearing epidermis and corium. The zone of proliferative growth bordering the ulcer is best shown under higher magnification (Fig. 4*C*). A thin layer of keratin covers the hyperplastic epithelium, which extends rather deeply into the underlying corium by means of proliferative rete pegs. The basal-cell layer, although showing some mitotic activity, is well delineated from the subjacent tissue and gives no evidence of anaplastic change. An increased vascularity is present in the corium, and there is some fibrosis as well as a diffuse infiltration by lymphocytes. Although the changes described are generally characteristic of the B complex–deficient group, many other animals demonstrate greater epithelial abnormality. Such a picture is illustrated in Fig. 4*D*, which shows basal-cell hyperactivity, loss of polarity, and appreciable variation in cellular size, shape, and staining qualities. Many of the cells are extremely large and irregular in outline, and contain bizarre nuclei, prominent nucleoli, and dense chromatin material.

That vitamin B complex deficiency contributes to the described lesions as a predisposing factor, and that the tobacco smoke per se serves as the irritating or exciting agent, can be demonstrated by the typically normal appearance of the untreated left ears of the experimental animals. Epidermis is seen as a thin, uniform layer covering both surfaces of the ear and overlying a rather narrow corium divided in its mid-region by a cartilaginous plate.

In determining which of the B complex fractions or components are related to the rather severe pathologic changes noted previously, it must be emphasized that the synergistic effects of the various deficiencies undoubtedly contribute to the over-all picture. However, riboflavin, pyridoxine, and pantothenic acid can be demonstrated as the most significant of the predisposing factors tested. Figs. 5 to 7 show typical examples of each of these deficiencies. Clearly evident are the areas of ulceration and scab formation bordered by thick, raised margins of indurated tissue. Histologically, the epithelium adjacent to zones of ulceration is hyperplastic and acanthotic, and often covered by keratin and cells undergoing parakeratotic change. The basal-cell region, while usually well demarcated from underlying corium, frequently shows mitotic activity, loss of polarity, and abnormalities in cellular size, shape, and staining quality. Contrasting with these changes is the normal-appearing histologic structure of the opposite, untreated ear, seen in all but two of the deficient animals (both members of the pyridoxine group).

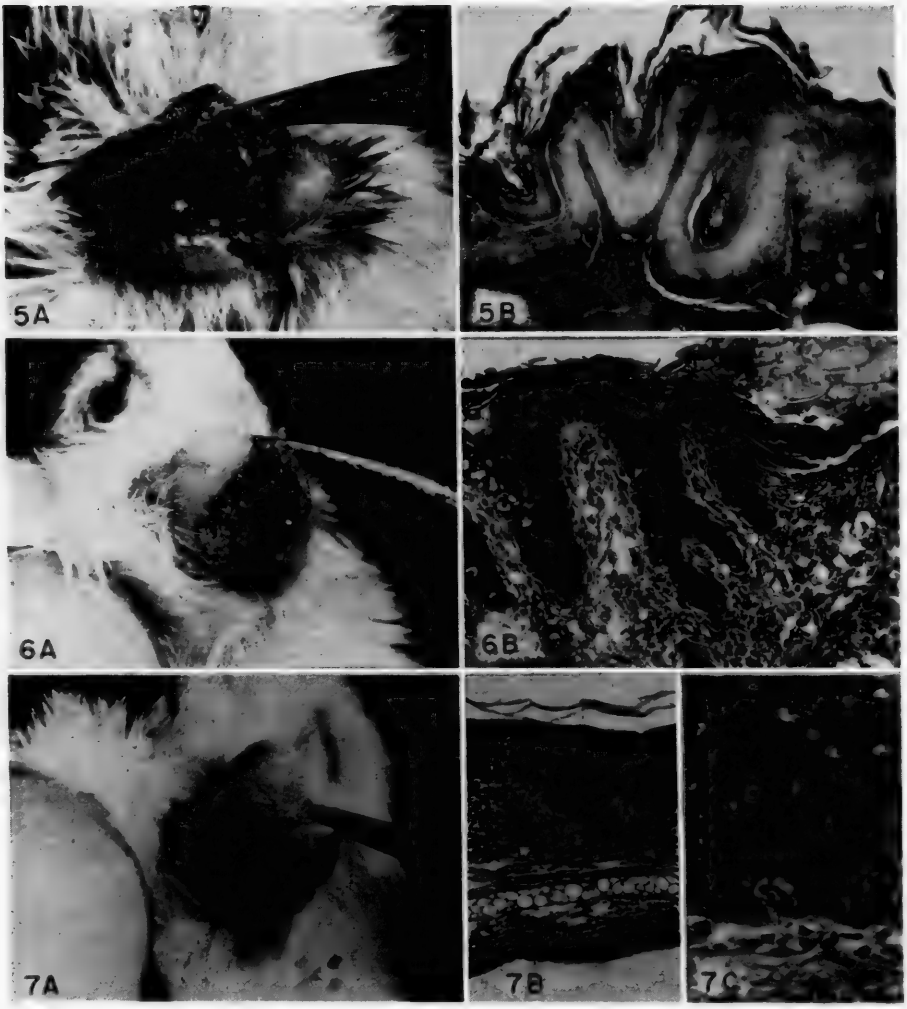

Fig. 5.—*A*, Ear of riboflavin-deficient animal; 20 smoke applications. There is irregular thickening, scab formation, and induration.

B, Riboflavin-deficient mouse; 40 smoke applications. A marked degree of keratinization, epidermal hyperplasia, and acanthosis is present. It is of interest to note that this biopsy was taken 5 months after terminating smoke applications.

Fig. 6.—*A*, Ear of pyridoxine-deficient mouse; 40 smoke applications. Note the area of ulceration bordered by a raised, rolled margin of indurated tissue.

B, Pyridoxine-deficient mouse. Histologic appearance after 36 smoke applications. Bordering the zone of ulceration is a markedly hyperplastic epithelium characterized by long, tentacle-like, rete pegs. The corium is edematous and infiltrated by numerous small round cells. A moderate degree of keratinization is present.

Fig. 7.—*A*, Ear of mouse maintained on a panthothenic acid–deficient diet; 40 smoke applications. Ulceration, scab formation, and induration are apparent.

B, Biopsy specimen from ear shown in *A*. Note the marked degree of epithelial hyperplasia and chronic inflammation of the corium. The contrasting appearance of the untreated side of the ear, inferior to the cartilaginous plate, is quite striking.

C, A high magnification of basal-cell region showing cellular change and mitotic activity.

Of the remaining vitamin B components evaluated in this study, namely thiamine, biotin, and niacin, none show any significant predisposing influence on tissue response to whole tobacco smoke. The gross and microscopic appearance of the treated ears is somewhat comparable to that observed in the normal diet group of animals.

Following the previously reported observation that castrated mice show a minimal tissue response to whole tobacco smoke regardless of whether they are on a normal or vitamin B complex–deficient diet,[1] it became of interest in the current study to re-evaluate these findings in gonadectomized, riboflavin-deficient animals. Although this was done in only 6 mice, it is of interest to note that the changes, while appreciably less than in the noncastrated groups, are greater than was anticipated. Gross and microscopic examinations show a moderate thickening of the smoked ear due principally to an increased keratinization and epithelial hyperplasia. These changes are quite similar to those seen in the normal diet animals (Fig. 3), although somewhat less marked.

DISCUSSION

Mice rendered deficient in the vitamin B complex show a far greater tissue susceptibility to whole tobacco smoke than do animals on a nutritionally adequate diet. The implication of riboflavin, pyridoxine, and pantothenic acid, as most significant in altering tissue response to tobacco irritation, is not surprising perhaps on the basis of their often demonstrated deficiency symptoms related to the skin. However, it should be re-emphasized that mice in the present study were maintained at such a level of deficiency as to assure relatively long periods of survival. Consequently, only 2 of the 70 animals comprising the riboflavin-, pyridoxine-, and pantothenic acid–deficient groups showed any evidence of ear changes referable to the deficiency per se and not associated with tobacco smoke exposures. It is conceivable that the absence of grossly and microscopically demonstrated skin changes in 68 of the deficient animals does not preclude the probability that the tissue is rendered more susceptible to such irritants as tobacco tar. Supporting this contention is the finding by Adams[6] which indicates that riboflavin deficiency in rats materially reduces oxygen consumption by the skin. Undoubtedly, this altered tissue function can significantly influence response to external environmental factors.

That thiamine-deficient animals show but slight ear pathosis following tobacco smoke exposures is perhaps explainable on the basis of their deficiency symptoms being referable principally to the nervous system and not the skin. Such an hypothesis is not applicable to the biotin group of mice, which failed to show any appreciable tissue change following exposure to tobacco smoke; this despite the fact that biotin deficiency symptoms are characteristically manifested in the skin. No explanation is offered for this apparent inconsistency.

The failure of the animals on niacin-deficient diets to demonstrate tissue changes greater than those observed in the normal diet group is not surprising in light of evidence that mice are able to synthesize nicotinic acid and consequently are not truly deficient in this vitamin B factor.[7]

Following the observation, previously reported, that gonadectomized mice on normal or B complex–deficient diets show an appreciable lessening of tissue response to whole tobacco smoke applications, it is of interest to note that the castrated riboflavin-deficient mice in the present study are not as completely free of pathologic change. However, since the abnormalities are comparable to those seen in the normal diet animals, the assumption appears valid that gonadectomy establishes an appreciable degree of tissue resistance. Further studies are in progress in order to attempt a clarification of this phenomenon, as well as to evaluate carefully any possible changes in the adrenal glands.

In an effort to determine whether the proliferative tissue changes observed in various of the vitamin-deficient animal groups are dependent upon continued exposure to tobacco smoke, a number of observations were carried out for several months after termination of applications. Since there was very little change in either the gross or the microscopic picture after 5 months, it would appear that the tissues do not assume an intrinsic capacity for unrestricted growth in this span of time. However, the histologic changes, specifically those related to cellular morphology, are consistent with that type of epidermal lesion often considered precancerous. Certainly it would seem quite significant that such relatively severe changes are produced in so short a period of time.

Although not described in the present report, it is of interest to interject an additional comment relative to findings in a large group of hamsters currently under study. These animals, divided into various experimental categories, are receiving whole tobacco smoke applications for a prolonged period. The sites of exposure are both oral mucosa and ear. At the end of 80 successive daily applications to vitamin B complex–deficient animals, there was practically no gross evidence of pathologic change in either of the treated regions. This indicates the important consideration that should be given to variations in response by different animal species as well as by different tissues, and poses the difficult problem of correlating findings in the experimental animal with observations in human beings.

SUMMARY AND CONCLUSIONS

By means of an apparatus designed to make applications of whole tobacco smoke to selected tissue sites, and on the basis of observations made on 201 Swiss strain mice divided into various control and vitamin-deficient categories, the following summary may be made.

1. Vitamin B complex deficiency in mice renders their cutaneous tissues more susceptible to the irritating effects of whole tobacco smoke. This confirms findings made in a previous report.

2. Riboflavin, pyridoxine, and pantothenic acid are the B complex components primarily responsible for such alterations in tissue response.

3. Animals maintained on diets deficient in biotin, thiamine, and nicotinic acid show no greater tissue susceptibility to tobacco smoke applications than do mice on a normal adequate diet. These observations are explainable, in part,

by the demonstrated resistance of mice to niacin deficiency, and by the fact that thiamine deficiency is manifested principally in noncutaneous tissues.

4. Gonadectomy renders cutaneous tissues less susceptible to the irritating effects of whole tobacco smoke.

5. Tissue response, under the conditions of the present study, is not influenced by sex.

6. The described cutaneous lesions are in striking contrast to the previously reported absence of pathologic change in oral tissues subjected to similar applications of whole tobacco smoke. This suggests either a basic difference in response by different tissues or a protective effect by the tongue and saliva in readily removing deposited tars.

7. The predominating feature of tissue response to whole tobacco smoke is a proliferative change characterized by hyperkeratosis, hyperplasia, acanthosis, and chronic inflammation of the corium. Cellular alterations in size, shape, and staining qualities as well as loss of basal-cell polarity and increased mitotic activity indicate the abnormal nature of the tissue change.

8. There is no evidence of an acquired capacity of the tissues for continued proliferative change after terminating exposures to the tobacco smoke.

9. Vitamin B complex–deficient hamsters, currently under study, show no significant cutaneous tissue changes even after receiving twice as many smoke applications as did the mice. This indicates the questionable validity of making conclusions on the basis of experiments with a single species of laboratory animal, and the speculation involved in applying such findings to man.

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ELECTRON MICROSCOPY OF HUMAN DENTIN

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BECAUSE both the organic and inorganic components are so finely divided in dentin as to be beyond the resolving power of a light microscope, electron microscopy is valuable according to Scott and Wyckoff,[1] who have reported their interesting results on replicas of fibrous components of human dentin. Pease[2] has succeeded in making ultrathin sections of decalcified rats' teeth. During our electron microscopic studies of dentin by means of methyl methacrylate-aluminum replicas, it has been shown to be possible to obtain satisfactory electron micrographs of Tomes's fiber, as well as its sheath, and Neumann's sheath which is enclosed by collagenous fibers. Moreover, the noncalcified fibers connecting the dentinal matrix and tubules have been detected in the dentin, which is not treated with acid.

Noncarious molars and premolars were immersed in 10 per cent Formalin immediately after extraction. The surfaces of cross and longitudinal slabs of dentin were polished with a whetstone. The surfaces required little additional polishing with metallographic papers. The surfaces of the slabs were etched for 10 seconds with 0.5 N HCl or for 2 minutes with 1.4 N HCl prior to replication. Other specimens were immediately replicated without polishing and etching. The replicas were made according to Tadano's methyl methacrylate-aluminum method.[3] The replicas were shadowed with chromium and examined with a Shimazu electron microscope. The reproductions presented in this paper have been selected from about 2,000 plates taken during the present experiments.

It has been difficult to demonstrate structural detail in the replicas of dentin slabs which are lightly etched for 10 seconds with 0.5 N HCl (Figs. 1 and 2). They have shown no fine detail other than the sheath of Tomes's fibers.

Fig. 3 shows that the sheath of Tomes's fibers remains attached to the replicas when the surfaces of longitudinal slabs of dentin are etched for 10 seconds with 0.5 N HCl. In these reproductions, we cannot find any difference in structure between the inside and the outside of the sheath. The sheath appears to be composed of thin, nonfibrous membranous tissue, and it is resistant to the strong acid in which it was immersed. In the replicas of cross slabs of dentin, the image, which is composed of both Tomes's fiber and its sheath, often can be seen (Fig. 4). These apparently are the structures remaining attached to the replica.

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Figs. 5 and 6 are replicas of longitudinal slabs of dentin which were etched for 2 minutes with 1.4 N HCl. These figures demonstrate the wall of dentinal tubule and the matrix. The wall is composed of large numbers of fibers which are more easily decalcified than the fibers in the dentinal matrix. The resemblance between these fibers and those obtained from tendons is very striking and the periodicity (⁀ 640A.) well defined. More of these fibers have been noted

Fig. 1.

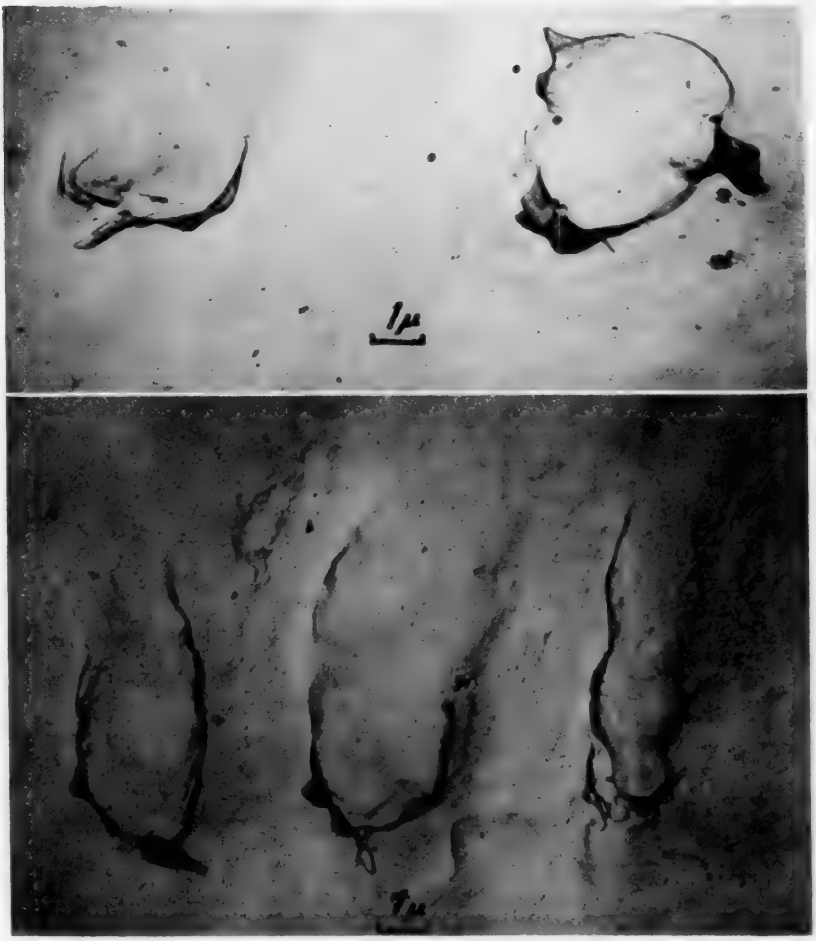

Fig. 2.

Figs. 1 and 2.—Human dentin etched 10 seconds with 0.5 N HCl, showing only the sheath of Tomes's fibers.

in longitudinal sections of the dentin than in cross sections, as seen in Figs. 5 to 7. The fiber bundlelike structures are found to run around the long axis of tubules.

Fig. 3.—Human dentin etched 10 seconds with 0.5 N HCl, showing the sheath of Tomes's fibers remaining attached to the replica.

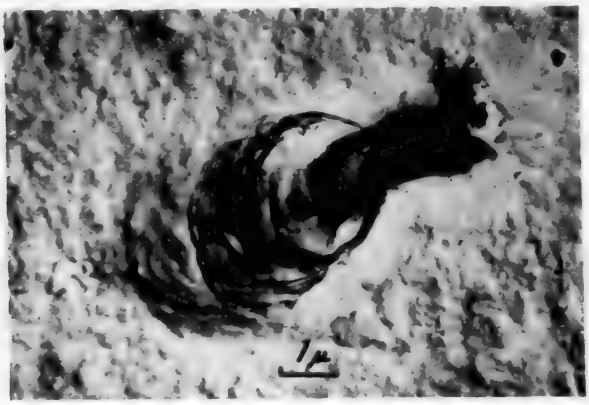

Fig. 4.—Human dentin etched 10 seconds with 0.5 N HCl, showing Tomes's fiber and its sheath.

Fig. 5.

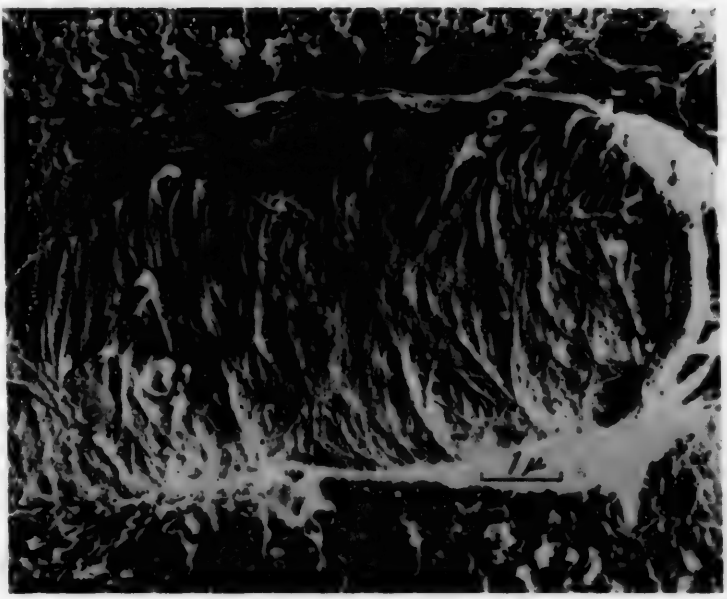

Fig. 6.

Figs. 5 and 6.—Longitudinal slabs of human dentin etched 2 minutes with 1.4 N HCl, showing the fibrous structure of the sheath of dentinal tubule (Neumann's sheath).

Fig. 7.—Cross slabs of human dentin etched 2 minutes with 1.4 N HCl, showing the fibrous structure of the dentinal matrix and surrounding the dentinal tubule.

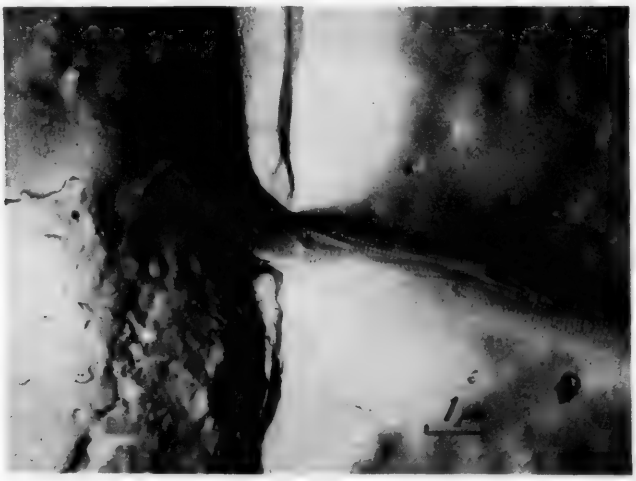

Fig. 8.—Human dentin without polishing and etching, showing the bundle of collagenous fibers in the dentinal matrix.

Fig. 9.—Human dentin without polishing and etching. The collagen fibers are found in the dentinal tubule. An arrow indicates the hole.

We have found the results interesting in the replicas of dentin slabs which are cracked in two with knife and hammer and viewed without being treated with any acid (Figs. 8 to 10). A thick bundle of noncalcified fibers is visible in the dentinal matrix (Fig. 8). These fibers are seldom found in the dentin of people older than 40 years of age. These noncalcified fibers enter the dentinal tubules through many holes which are found on the wall of these tubules. As soon as a fiber bundle enters the tubule, the fiber bundle resolves itself into a multiple of fibers (Fig. 9).

Fig. 10.—Human dentin without polishing and etching. The noncalcified fibers enter the dentinal tubule through many holes which are found on the wall of this tubule. Arrows indicate the holes.

SUMMARY

1. Metal-shadowed methyl methacrylate-aluminum replicas have proved to be profitable for the study of fibrous components of human dentin.

2. The sheath of Tomes's fibers is composed of a thin, nonfibrous membrane.

3. When the surfaces of slabs of dentin are etched with 1.4 N HCl for 2 minutes, Neumann's sheath, which is enclosed with collagenous fibers, becomes clearly visible.

4. Noncalcified, collagenous fibers have been found in the matrix of mature dentin and in the dentinal tubule.

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STUDIES OF THE COMPOSITION OF TEETH

II. Spectrochemical Analysis of Enamel and Dentin From Syrian Hamsters

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A FEW incomplete spectrochemical analyses have been made of calcified tooth tissues. Fox and Ramage[1] included teeth in the list of tissues and organs studied spectroscopically but did not report on their composition in detail. Boissevain and Drea[2] made spectrograms of teeth, especially for their fluorine content, while Drea[3] also investigated teeth spectrographically. Lowater and Murray[4] made qualitative spectrograms of human enamel and dentin, dog dentin, and the anterior teeth of rats with special emphasis on the analysis of fluorine, chlorine, potassium, iron, copper, and zinc. Murray, Glock, and Lowater[5] made spectrographic determinations of iron in teeth. None of these investigators has applied quantitative spectrographic methods to the analysis of the tissues of the tooth. In previous studies of the composition of teeth, we have analyzed ashed enamel and ashed dentin from the incisor and molar teeth of adult Syrian hamsters for their principal inorganic constituents, calcium, phosphorus, and magnesium.[6] Since very little is known of the other elements in such tissues, quantitative spectrochemical analyses were made of separated enamel and dentin from adult Syrian hamsters for 34 elements that might be present. The results obtained in this study are the basis of the following report.

EXPERIMENTAL PROCEDURE

Enamel and dentin from Syrian hamsters which were analyzed by spectrochemical methods were samples from tissues which had been analyzed previously by wet chemical methods.[6] The teeth from 53 males and 63 females were obtained as described[6] and divided into groups of anterior and posterior teeth from males and females. After each of the 8 groups of teeth were extracted separately with ethyl alcohol and ethyl ether for 12 hours each, they were dried in air at 100° C. to a constant weight. The extraction of the teeth with fat solvents facilitated their handling during their subsequent separation into enamel and dentin. Each group of teeth was ground in a mullite mortar until the particles passed a 200-mesh nylon screen. The ground teeth were again dried to a constant weight at 100° C., after which the enamel and dentin were

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separated by Gilda's modification of the Manly-Hodge flotation technique[7] until each tissue was at least 98 per cent pure when analyzed by the method of Manly and Hodge.[8] The separated tissues were then dehydrated at 100° C. to a constant weight, after which they were ashed by heating at 900° C. until a constant weight was attained. The ashed tissues, whose weight usually ranged from 50 to 100 mg. and which also contained the inorganic portions of the organic matrix, were then analyzed for 34 elements, utilizing a Jarrel-Ash grating spectrograph in the Spectrochemistry Section of the Division of Chemistry of the National Bureau of Standards.

The technic of using the emission spectrograph for the analysis of trace elements has the advantage of determining the presence or absence of many elements simultaneously in true concentrations as low as 1 per 1,000 μg. More importantly for this study, it may be used for quantitative analyses of many such elements. If a sample to be analyzed is burned in the carbon arc of the Jarrel-Ash spectrograph, the atoms present are excited to a high electronic energy state followed by emission of light as the excited atoms return to lower energy states, or when ionization takes place by the complete ejection of an electron from the atom. The light emitted is of specific wave lengths, characteristic of the elements, and appears as spectrum lines when viewed through a suitable prism. When the light emitted from one or several elements is introduced into the slit of the spectrograph and allowed to impinge upon a diffraction grating, the light is dispersed into its component wave lengths, which may then be recorded on photographic film in the form of lines. Known concentrations of some element may be run for film calibration when analyzing samples quantitatively. The lines on the film can be measured accurately with a microdensitometer, thus giving the basis for quantitative analysis.[9] The deviation for each element in this type of analysis is about ± 5.0 per cent, being more accurate when the elements are in low concentrations. Each sample of tooth tissue was analyzed 3 times and the value obtained expressed as an average of the 3 analyses. The region of the spectrum utilized was 2,200 to 4,800 Å, except for 4 alkali elements. The 6,400 to 8,800 Å region of the spectrum was utilized for analysis of the 4 alkali elements, cesium, potassium, lithium, and rubidium.

RESULTS

The results of the quantitative spectrochemical analysis of incisor and molar enamel from male and female Syrian hamsters are shown in Table I. All values are expressed as a percentage of the ashed sample. At the end of the table, the ratio of each ashed sample to the original dehydrated tissue is given as a conversion factor, which, when multiplied by the percentage of the elements in ashed tissues, gives the percentage of that element in whole dehydrated tissue. Elements that fall outside the range of present standards and those for which accurate measurements could not be made are reported as estimated concentrations. Seventeen elements were found consistently in the enamel of the hamster, while 2 additional elements were found occasionally. All the samples of enamel contained calcium and phosphorus as the major

constituents, with magnesium, sodium, and iron as the only other minor constituents observed in concentrations above 0.1 per cent. Gold, beryllium, bismuth, cadmium, cobalt, cesium, gallium, germanium, indium, molybdenum, rubidium, antimony, titanium, zinc, and zirconium were not found within the range of the estimated limit of detection. Nickel and tin were not found consistently in all tissues. Iron was the element that varied most between incisor and molar enamel. Lesser variations of some of the other elements occurred between similar tissues from incisor and molar teeth and between tissues obtained from males and females. Lead was the only element which did not give consistent values when different portions of a sample were tested.

TABLE I
SPECTROCHEMICAL ANALYSIS OF ENAMEL OF SYRIAN HAMSTER. CONCENTRATIONS EXPRESSED AS A PERCENTAGE OF THE ASHED SAMPLE

ELEMENTS	♂ INCISOR ENAMEL	♀ INCISOR ENAMEL	♂ MOLAR ENAMEL	♀ MOLAR ENAMEL	ESTIMATED LIMIT OF DETECTION
Ag	L0.0005	L0.0005	L0.0005	L0.0001	0.00001
Al	L0.002	0.002	L0.002	L0.002	0.001
Au	—	—	—	—	0.001
B	L0.002	L0.002	L0.002	L0.002	0.0005
Ba	a0.015	a0.015	a0.015	a0.015	
Be	—	—	—	—	0.00001
Bi	—	—	—	—	0.001
Ca	VS	VS	VS	VS	
Cd	—	—	—	—	0.005
Co	—	—	—	—	0.003
Cr	a0.003	a0.003	a0.003	a0.001	0.0001
Cs	—	—	—	—	0.001
Cu	0.004	L0.001	0.003	L0.001	0.00001
Fe	G0.1	G0.1	L0.003	L0.003	0.00005
Ga	—	—	—	—	0.001
Ge	—	—	—	—	0.001
In	—	—	—	—	0.002
K	0.02	0.02	0.01	0.01	0.0001
Li	0.001	0.001	0.001	L0.0005	0.0001
Mg	0.19	0.20	0.40	0.35	
Mn	0.00015	0.002	0.010	0.013	0.0005
Mo	—	—	—	—	0.0005
Na	G0.5	G0.5	G0.5	G0.5	
Ni	—?	—?	0.002	0.002	0.001
P	VS	VS	VS	VS	
Pb*	0.005	0.03	L0.0001	L0.0001	0.00005
Rb	—	—	—	—	0.001
Sb	—	—	—	—	0.002
Si	L0.01	0.03	0.02	L0.01	0.002
Sn	L0.002	—?	L0.001	—?	0.0005
Sr	a0.02	a0.02	a0.02	a0.02	
Ti	—	—	—	—	0.05
Zn	—	—	—	—	0.05
Zr	—	—	—	—	0.05
Conversion factor	0.89	0.85	0.92	0.89	

Factor × per cent = per cent of element in whole tissue

Legend: VS, over 10 per cent; G, greater than; L, less than; —, not detected; a, approximately.

*Lead is the only element which did not give consistent values when different portions of a sample were tested.

The results of the spectrochemical analysis of incisor and molar dentin from male and female Syrian hamsters are shown in Table II. The values for the

elements in dentin are expressed as in Table I, with the factors for the ratio of each ashed sample to the original dehydrated tissue given at the end of the table. The estimated limit of detection of each element is expressed as in Table I. The elements which fall outside the range of present standards and those for which accurate measurements could not be made are reported as estimates or ranges of concentration. All the elements not detected in enamel were not detected in dentin; in addition, nickel and chromium were not detected consistently in either incisor or molar dentin. Cesium was detected only

TABLE II

SPECTROCHEMICAL ANALYSIS OF DENTIN OF SYRIAN HAMSTER. CONCENTRATIONS EXPRESSED AS A PERCENTAGE OF THE ASHED SAMPLE

ELEMENTS	♂ INCISOR DENTIN	♀ INCISOR DENTIN	♂ MOLAR DENTIN	♀ MOLAR DENTIN	ESTIMATED LIMIT OF DETECTION
Ag	0.01	0.02	0.01	0.002	0.00001
Al	0.003	L0.002	0.004	L0.002	0.001
Au	—	—	—	—	0.001
B	L0.005	L0.002	L0.005	L0.002	0.0005
Ba	a0.015	a0.015	a0.015	a0.015	
Be	—	—	—	—	0.00001
Bi	—	—	—	—	0.001
Ca	VS	VS	VS	VS	
Cd	—	—	—	—	0.005
Co	—	—	—	—	0.003
Cr	—?	—?	a0.0005	—?	0.0001
Cs	—	—	a0.0005	—?	0.0001
Cu	L0.001	0.002	0.003	L0.001	0.0001
Fe	0.01	0.006	L0.003	L0.003	0.0005
Ga	—	—	—	—	0.001
Ge	—	—	—	—	0.001
In	—	—	—	—	0.002
K	0.04	0.05	0.02	0.02	0.0001
Li	L0.0005	L0.0005	L0.0005	L0.0005	0.0001
Mg	G0.8	G0.8	0.60	0.56	
Mn	0.0015	L0.001	0.0049	0.0041	0.0005
Mo	—	—	—	—	0.0005
Na	G0.5	G0.5	G0.5	G0.5	
Ni	—?	—?	—?	—?	0.001
P	VS	VS	VS	VS	
Pb*	0.015	—?	L0.0001	L0.0001	0.00005
Rb	—	—	—	—	0.001
Sb	—	—	—	—	0.002
Si	0.02	L0.01	L0.01	L0.01	0.002
Sn	L0.001	L0.001	—?	—?	0.0005
Sr	a0.02	a0.02	a0.02	a0.02	
Ti	—	—	—	—	0.05
Zn	—	—	—	—	0.05
Zr	—	—	—	—	0.05
Conversion factor	0.76	0.75	0.74	0.74	

Factor × per cent = per cent of element in whole tissue

Legend: VS, over 10 per cent; G, greater than; L, less than; —, not detected; a, approximately.

*Lead is the only element which did not give consistent values when different portions of a sample were tested.

in molar dentin from males, while lead was not detected in incisor dentin from females. Tin was not detected in molar dentin from either males or females. The iron content of incisor and molar dentin was less than that of incisor and

molar enamel, and the iron content of incisor dentin was more than that of molar dentin. The iron content of incisor enamel and dentin from males was greater than that of similar tissues from females.

DISCUSSION

In studying the chemical composition of teeth, attention has been directed generally toward ascertaining the more abundant inorganic elements in enamel and dentin, with a view to defining the chemical structure of the inorganic phases of these tissues or to ascertaining some variation in composition between carious or noncarious teeth.[10] Even today, the basic chemical formula of the inorganic phases of enamel and dentin has not been completely defined, and our knowledge of the trace elements present in the calcified tissues of the tooth is insufficient for their consideration when attempting to devise such formulas, or to ascertain the relationship of such elements to the inorganic or organic phases of enamel and dentin. In studies of the relationship of the composition of teeth to caries susceptibility, the trace element fluorine is the only one that has been shown to influence consistently the resistance of teeth to dental caries. The function of other trace elements and their influence, if any, on the physical and chemical properties of tooth tissues is undefined.[10]

In an attempt to increase our knowledge of the trace elements of calcified tooth tissues, we have analyzed hamster enamel and dentin for 34 possible trace elements, of which 17 were found consistently in enamel and 2 additional elements were found occasionally. Sixteen elements were found consistently in dentin and 3 were present occasionally. Composition differences were observed in enamel and dentin for chromium, iron, magnesium, manganese, nickel, and silver. The other elements were not detected or did not vary significantly with the source of the tissue. Iron was much more abundant in the calcified tissues that are normally pigmented, such as incisor enamel. Higher concentrations of iron were present in incisor dentin than were present in molar dentin, although some iron was present in all samples of enamel and dentin. Zinc, which has been found previously in teeth in concentrations of about 0.02 per cent,[11] was not found in either enamel or dentin by spectrographic analysis. No significance can be attached to the absence of zinc since the estimated limit of detection by this method of analysis is about 0.05 per cent.

The magnesium detected in enamel and dentin by spectrochemical analysis was lower than that previously detected by chemical analysis. While the accuracy of the spectrochemical method of analysis usually decreases as the concentration of the element increases, the reason for the spectrochemical method detecting less magnesium is as yet unknown. Lead was the only element which did not give consistent values when several analyses were made of one sample of tissue.

The ashed enamel and dentin analyzed spectrochemically in this study contained the inorganic elements of both the inorganic and organic phases of the tissues. Many of the trace elements that have been detected in the ash from

whole tissues may decrease in concentration or be absent when the organic matrix of enamel and dentin is removed prior to analyzing. Enamel and dentin whose organic matrices were removed prior to spectrochemical analysis are being analyzed spectrochemically.

SUMMARY

Enamel and dentin from incisor and molar teeth of adult male and female Syrian hamsters were analyzed spectrochemically for 34 elements, of which 17 were found consistently in enamel and 16 were found consistently in dentin. Several other elements were found occasionally in the tissues. Calcium and phosphorus were the major constituents, while manganese, sodium, and iron were the only other elements that were above 0.1 per cent. Composition differences were noted for chromium, iron, magnesium, manganese, nickel, and silver.

We wish to express our appreciation to the Spectrochemistry Section of the Division of Chemistry of the National Bureau of Standards where the spectrochemical analysis was accomplished.

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THE IDENTIFICATION OF PYRIMIDINES IN THE FLUORESCING FRACTIONS OF THE TEETH OF THE SPERM WHALE (PHYSETER MACROCEPHALUS)

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WE HAVE earlier reported on the fluorescence of human teeth when irradiated with ultraviolet light.[6] During the course of this work we were fortunate enough to obtain a few teeth of the sperm whale (*Physeter macrocephalus*). These teeth are characterized by a complete absence of enamel and consist of a central core of dentin surrounded by a thick layer of cementum. The incisal tip of the tooth consists of exposed dentin. Each tooth was about 5 or 6 inches in length and weighed between 400 and 650 Grams. In view of the relatively large amount of material available, it was decided to investigate the fluorescing fractions of these teeth.

EXPERIMENTAL

The methods of exciting and examining fluorescence were the same as those described earlier.[6] The cementum and dentin were separated by cleavage, and any small portions of either tissue which remained were removed with the aid of a grinding wheel.

Whale Cementum.—The cementum was dull white in color and fluoresced strongly with a blue color. Its specific gravity was about 1.9; the nitrogen content averaged 3 per cent (w/w).

Extraction of lipids: The cementum was broken into small pieces in a percussion mortar and dried at 100° C. A sample (15.393 Gm.) was extracted with ether in a continuous extractor for 6 hours; this yielded 0.031 Gm. of lipid material (0.20 per cent w/w) having a weak blue fluorescence both in solution and in the solid state. This lipid fraction was saponified with 60 per cent (w/v) NaOH. The nonsaponifiable matter, when dissolved in light petroleum, fluoresced weakly blue and gave a faintly positive Liebermann-Burchard reaction, indicating the presence of a sterol. Adsorption chromatography on activated alumina resolved 2 fractions. Ultraviolet absorption spectra of these fractions did not reveal any selective absorption. The saponifiable matter was acidified with H_2SO_4 and the acidic materials extracted with ether. A faintly positive molybdate test for phosphate suggested the presence of phospholipid. Adsorption chromatography on alumina yielded only one fluorescing fraction; its ultraviolet absorption spectrum showed a small inflexion in the region of 240 to 270 mμ. The quantity of fluorescing materials present in these fractions was very small and their study was not pursued.

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Digestion of cementum with ethylene glycol containing 3 per cent (w/v) KOH: Samples (about 200 Gm.) were broken into small pieces and extracted by refluxing with 300 ml. of the alkaline ethylene glycol. After 4 hours the dark red-brown liquor was decanted, the pieces of cementum crushed (this was rendered much easier after digestion), and the extraction continued with the same volume of 3 per cent (w/v) aqueous KOH. Both extracts were mixed, cooled, and extracted with 4 successive 50 ml. portions of ether. The ether extracts, containing nonsaponifiable matter, were evaporated to dryness and extracted with light petroleum, material insoluble in this solvent being dissolved in ether. The 2 fractions were named WC1 and WC2, respectively. After ether extraction, the aqueous glycol liquor was acidified with H_2SO_4 and continuously extracted with ether in a liquid/liquid extractor. The extracts were evaporated and extracted with light petroleum, petrol-insoluble matter then being dissolved in ether. These 2 solutions were termed WC3 and WC4, respectively.

All 4 fractions exhibited blue fluorescence in ultraviolet light and were colored yellow. It was noticeable that fraction WC4 contained most dissolved material, was the most pigmented, and fluoresced the most strongly. The nature of the materials in these fractions was probably of 3 types: (a) true lipids; (b) nonlipid material partially soluble in ether; and (c) substances carried over in the wet ether.

Fractions WC1, WC2, and WC3 contained very little material; attempts to resolve them by adsorption chromatography, although giving some qualitative separation, yielded such tiny fractions that their study was abandoned in favor of the much larger fraction WC4.

Adsorption chromatography of fraction WC4: The ethereal solution, containing acidic or potentially acidic substances, was chromatographed on a column of activated alumina (Grade O, Peter Spence and Co., Ltd., Widnes). The strongly fluorescing material was adsorbed firmly at the top of the column, and development with ether caused elution of a faintly fluorescent fraction. Ether methanol (1:1 v/v) eluted several small fractions, and methanol alone 1 or 2 more tiny fractions (WC4/1 to WC4/7). The main bulk of the fluorescing material, however, could be removed only by development with methanol ammonia (1:1 v/v). This fraction moved as a discrete band and was termed WC4/8; from its behavior on the column we considered that it was not a lipid and was acidic in nature.

The ultraviolet absorption spectra of the earlier fractions showed little selective absorption except in the case of WC4/6. This small fraction, developed from the column with methanol, fluoresced with a marked purple color; the ultraviolet absorption curve had $\lambda_{max.}$ 250 mμ, and 345 mμ, $\lambda_{min.}$ 295 mμ. The extinction at 250 mμ being 1.4 times that at 345 mμ. From the appearance of the absorption curve this fraction, although small, was quite pure. Unfortunately, the fraction was so tiny that further study was not possible at this stage.

Fraction WC4/8: The eighth and final fraction from WC4 was by far the largest one obtained. We decided therefore to concentrate our efforts on its investigation. A further 200 Gm. of cementum was treated as before and

the final fraction of WC4, henceforth known as compound R, was obtained by chromatographic separation on an alumina column. The fraction was purified by a further chromatographic treatment. About 10 mg. of an amorphous material was obtained. The absorption spectrum of a solution in ethanol showed a well-defined maximum at 264 mμ and a minimum at 230 mμ. Fig. 1 shows the ultraviolet absorption curves in 0.1N HCl and in 0.1N NaOH.

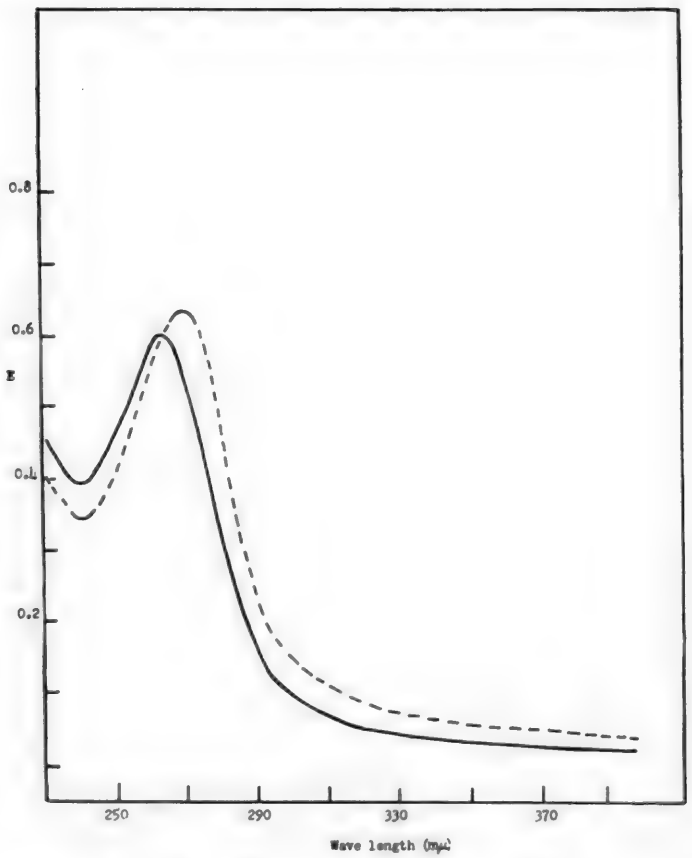


Fig. 1.—Absorption spectra of compound R obtained from whale cementum; ----- in 0.1 NHCl; ——— in 0.1N NaOH.

In earlier work,[6] it was suspected that pyrimidines were present in fractions from human teeth. The absorption spectrum of compound R is similar to that of pyrimidines, so we decided to investigate this possibility. Nitrogen determinations on compound R gave values of about 22 per cent. The following chemical tests were made: Benedict's test for reducing sugar was negative; ninhydrin test was negative; Ehrlich's reagent gave a faintly positive test; and Wheeler and Johnson's test was negative (this test is given by uracil and cystosine but not by thymine). Compound R could be readily acetylated, using acetic anhydride and pyridine, its weight being thereby increased by about 40

per cent. It could not be methylated by the use of diazomethane. The fluorescence in neutral solution (ethanol or water) was blue, in dilute NaOH it was blue-green, but was yellow in the presence of ammonia. The absorption spectra of purines and pyrimidines have received considerable attention.[2, 3, 8, 12, 15, 16] Comparing our results with those of these workers we concluded that: (1) compound R was probably a pyrimidine rather than a purine; (2) the compound (if a pyrimidine) was substituted in the 2, 6; 4, 6; or 2, 4, 6 positions, to account for the selective absorption with $\lambda_{max.}$ 265 mμ; (3) there was no amino group present since we did not obtain a precipitate with phosphotungstic acid; (4) the compound appears to be relatively symmetrical about the 2, 5 positions; otherwise there should be a large shift in absorption maximum at an alkaline pH; (5) if no amino group is present, a hydroxy or keto group capable of enolization must be present to account for acetylation; (6) the compound may be derived from a weakly acidic substance which has been eluted from the alumina column as an ammonium salt. Many of the properties could be ascribed to uracil (2, 6-dioxypyrimidine) or thymine (2, 6-dioxy-5-methylpyrimidine), the latter, according to Levene and Bass,[10] differs from other common pyrimidines in being slightly soluble in ether. However, the fact that the absorption spectrum in alkaline solution showed practically the same maximmum as in neutral and acid solution seemed to negate this possibility. It was decided to apply classical chemical methods for the separation of purines and pyrimidines to compound R. A further sample of R was therefore prepared from 200 Gm. cementum.

Chemical procedure for the separation of purines and pyrimidines: The procedure was based on that outlined by Levene and Bass.[10] An aqueous solution of compound R was treated with twice its volume of ammonia (0.88 s.g.*). No precipitate was formed, which indicated that guanine was absent. The addition of phosphotungstic acid did not cause any precipitation; therefore, no basic compounds such as adenine or cystosine were present. The excess of phosphotungstic acid was removed by boiling with $Ca(OH)_2$ and filtering off the solids. The basic filtrate, which was still strongly fluorescent, was treated with $AgNO_3$ solution until precipitation was complete. The precipitate was collected, washed, suspended in water and decomposed by passing H_2S through the suspension for one hour. The silver sulfide was removed by filtration, the filtrate freed of H_2S by aeration, and evaporated to dryness in vacuo. The yellow gum thus obtained was dissolved in ethanol and referred to as "compound S." The absorption spectrum in 0.1N NaOH had $\lambda_{max.}$ 260-262 mμ, $\lambda_{min.}$ 238 mμ; in 0.1N HCl, $\lambda_{max.}$ 265-267 mμ, $\lambda_{min.}$ 242 mμ; in addition there was another small peak, $\lambda_{max.}$ 325 mμ, $\lambda_{min.}$ 305 mμ.

The yellow gum (30 mg.) was dissolved in methanol/ether (1:1, v/v) and chromatographed on a column of $CaCO_3$. A small portion of weakly fluorescent material was not adsorbed; this was discarded. The main fraction was collected and termed "S_2." Its absorption spectrum in 0.1N HCl had $\lambda_{max.}$ 267 mμ, $\lambda_{min.}$ 243 mμ, but the smaller peak present in S at 325 mμ had disappeared. The solution of S_2 was evaporated yielding 8 mg. of material which contained 13 per cent N (micro-Kjeldahl).

*Specific gravity.

Comparison between R and S_2: The two fractions appeared to be very similar in spectroscopic and fluorescent properties. The only fundamental difference was in nitrogen content and pH in solution. R contained 22 per cent N and was neutral in reaction. S_2 contained 13 per cent N and was slightly acidic. This could perhaps be explained if R were the ammonium salt of S_2. If S_2 contained only 13 per cent N, and yet was a pyrimidine derivative, so that it must contain at least 2 nitrogen atoms, its minimum molecular weight would be of the order 200 to 250. This suggested that there must be a substituent group in the pyrimidine ring.

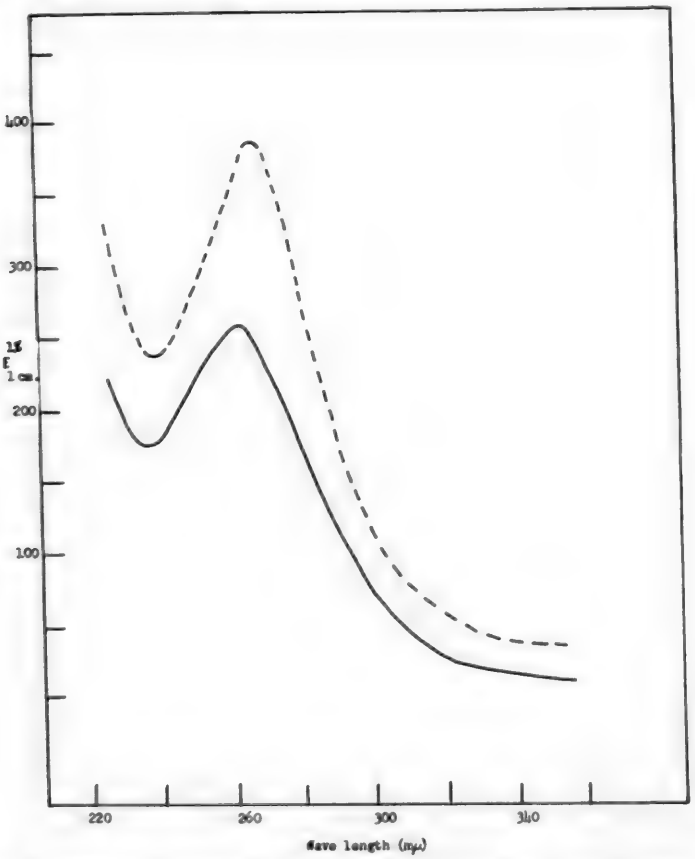


Fig. 2.—Absorption spectra of compound S_2 obtained from whale cementum; ------ in 0.1N HCl; ——— in 0.1N NaOH. Maximum absorption does not shift to longer wave length in alkaline solution; analogous to thymine deoxyriboside.

A further supply of S_2 was prepared, 250 Gm. of cementum yielding 35 mg. of yellow gum. The absorption spectra data were as follows: in pH 7 buffer, $E^{1\%}_{1\,cm.}$ 375 at $\lambda_{max.}$ 264 mμ, $\lambda_{min.}$ 237 mμ; in 0.1N HCl $E^{1\%}_{1\,cm.}$ 386 at $\lambda_{max.}$ 268 mμ, $\lambda_{min.}$ 239 mμ; in 0.1N NaOH, $E^{1\%}_{1\,cm.}$ 257 at λ_{max} 263 mμ, $\lambda_{min.}$ 236 mμ. There is thus no great shift in the wave length of maximum absorption in alkaline solution (Fig. 2).

If S_2 were a simple pyrimidine, this would suggest symmetry in the molecule. Thus, barbituric acid (2, 4, 6-trioxypyrimidine) exhibits no shift with pH change,[8] whereas the asymmetrical uracil (2, 6-dioxypyrimidine) has a maximum absorption in acid at 252 mμ and 285 mμ in alkaline solution.[15] The absorption spectrum of S_2 does not resemble that of barbituric acid, but it does resemble that of 5-methylbarbituric acid.[7, 17] However, this compound is unstable in acid solution, the extinction value decreasing to one-fifteenth of its original on standing 16 hours at pH 2. No significant decrease in the absorption of S_2 could be demonstrated in these circumstances.

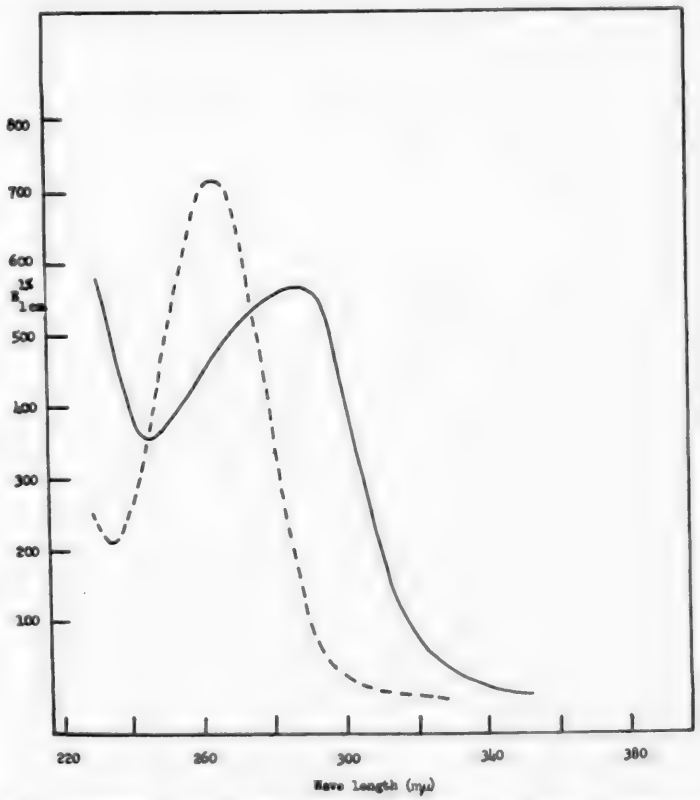


Fig. 3.—Absorption spectra of compound T obtained from whale cementum; - - - - - in 0.1N HCl; ——— in 0.1N NaOH. Note shift of maximum absorption to longer wave length in alkaline solution; analogous to thymine.

Stimson and Reuter[16] observed that the substitution of groups on the nitrogen atom at position 3 stabilized the molecule of an asymmetrical pyrimidine, so that its absorption maximum in alkaline solution does not show the normal shift to longer wave length. They refer specifically to thymine desoxyriboside, which possesses the following spectroscopic data, in acid and neutral solution, $\lambda_{max.}$ 265 mμ ($\epsilon_{max.}$ 9,000); in alkaline solution, $\lambda_{max.}$ 265 mμ ($\epsilon_{max.}$ 6,500). Macnutt[11] gave rather higher values, 9,500 and 7,300, respectively. In this compound, the group substituting in position 3 does not absorb in the ultraviolet

region. A particularly noticeable feature is the decrease in extinction coefficient by one-third in alkaline solution. If we examine the data for S_2 we find that this reduction also occurs; $E^{1\%}_{1\,cm.}$ values are reduced from 380 to 257. It would appear, therefore, that S_2 is a pyrimidine, probably thymine, which carries a nonabsorbing substituent group at position 3; such a group, if present, must have resisted hydrolysis in the treatment with alkaline ethylene glycol.

In order to test our hypothesis, and to split off any side chain, a sample of S_2 was hydrolyzed by refluxing with 6N HCl for four hours. The solution was evaporated to dryness, redissolved in ethanol, and evaporated several times to remove traces of HCl. Finally, the solid material was dissolved in a few milliliters of 0.1N NaOH and twice the volume of ethanol added. After the solution was kept for 3 days at 0° C., small feathery crystals formed and were collected by filtration. This very small quantity of crystalline material was termed "compound T." It was dissolved in 0.1N NaOH and the absorption spectrum determined before and after acidifying to pH 1. The spectroscopic data for compound T and thymine were as follows:

	Compound T		*Thymine*	
	$\lambda_{max.}$	$\lambda_{min.}$	$\lambda_{max.}$	$\lambda_{min.}$
pH 1	264 mμ	235 mμ	263-265 mμ	235 mμ
pH 13	290 mμ	245 mμ	290 mμ	245 mμ

Thus, compound T is spectroscopically very similar to an authentic sample of thymine, melting point 318° C. (L. Light and Co.).

A further sample of compound T (10.2 mg. of crystals, melting point 315-317° d.) was prepared from 250 Gm. of cementum. Solutions (0.001 per cent w/v) were prepared in 0.1N HCl and in 0.1N NaOH. The curves are shown in Fig. 3.

In 0.1N HCl and 0.1N NaOH, $E^{1\%}_{1\,cm.}$ was 720 and 570, respectively. This shift of maximum absorption in alkaline solution to longer wave length is exactly as for thymine. Assuming that compound T is thymine, $\epsilon_{max.}$ would be 9,072 in acid and 7,182 in alkaline solution, rather higher than the accepted values for thymine, which are 8,000 and 5,200, respectively. We are unable to explain this discrepancy at present; it may, however, be due to the presence of a small amount of another pyrimidine which absorbs selectively in the same region as thymine but with greater intensity.

Infrared spectroscopy: To gain additional information concerning the identity of compound T, use was made of infrared spectroscopy rather than elemental analysis; the spectrum was compared with that of an authentic sample of thymine. The solids were dispersed in Nujol (liquid paraffin) and examined on the infrared spectrometer. The results are given in Table I.

From the results shown in Table I it is apparent that the 2 spectra are identical with respect to all points where strong peaks are found. We consider, therefore, that compound T is indeed thymine (2, 6-oxy-5-methylpyrimidine).

Whale Dentin.—The dentin is harder than the cementum. It possesses a pinkish brown color contrasting with the dull white of the cementum, and fluoresces with a much whiter light. It contains 3.02 per cent (w/v) N compared with 3.00 per cent (w/v) N in cementum.

Extraction of lipids: Dentin (3.024 Gm.) was powdered in a percussion mortar and extracted with ether for 6 hours in a continuous extractor. Lipid material obtained weighed 0.021 Gm. (0.7 per cent w/w) and exhibited feeble fluorescence. After saponification, the nonsaponifiable matter gave a weak sterol reaction. The study of these lipids was not pursued.

TABLE I
THE COMPARISON OF INFRARED SPECTRA OF THYMINE AND COMPOUND T

THYMINE		COMPOUND T		
WAVE NUMBER ($CM.^{-1}$)	INTENSITY	WAVE NUMBER ($CM.^{-1}$)	INTENSITY	GROUP INDICATED
3,125	Strong	3,125	Strong	NH
2,930	Strong	2,932	Strong	$C-CH_3$
2,849	Strong	2,849	Strong	C–H
1,724	Strong	1,724	Strong	C=O
1,675	Strong	1,675	Strong	C=N–(?)
1,431	Average	1,431	Average	R–CH=CH–R
1,366	Average	1,366	Average	

The spectra were interpreted with reference to Gilman.[5]

Digestion of dentin with alkaline ethylene glycol: Dentin (250 Gm.) was treated exactly as was the cementum. Extraction of the digest with ether before and after acidification yielded fluorescent fractions very much smaller indeed than those obtained from cementum. A fraction WD4 was prepared corresponding to WC4 and subjected to chromatographic analysis on an alumina column. The final fraction eluted with methanol/ammonia (1:1, v/v) appeared very similar to compound R, but there was very much less of it.

A further small quantity of this fraction was prepared from about 500 Gm. of dentin and treated as for the preparation of compound S_2. A small amount of a dark red gum was obtained. The spectroscopic data were as follows: in 0.1N HCl, $\lambda_{max.}$ 268 mμ, $\lambda_{min.}$ 240 mμ, $E^{1\%}_{1\,cm.}$ 265; in 0.1N NaOH, $\lambda_{max.}$ 263 mμ, $\lambda_{min.}$ 238 mμ, $E^{1\%}_{1\,cm.}$ 175. Thus, it is very similar to S_2. The fraction was hydrolyzed with 6N HCl for 6 hours, taken to dryness, and dissolved in hot water. This aqueous solution was thoroughly extracted with ether. The ether extracts were evaporated and redissolved in water, the solution was concentrated and left to stand at 0° C. overnight, but no crystals were obtained. The absorption spectra of the solution were similar to those of thymine: at pH 13, $\lambda_{max.}$ 288-290 mμ, $\lambda_{min.}$ 244 mμ; at pH 1, $\lambda_{max.}$ 263-265 mμ, $\lambda_{min.}$ 236 mμ. It was impossible to carry out satisfactory infrared spectroscopy as no crystalline material capable of dispersion in Nujol was obtained. Nevertheless, it appears highly probable that thymine also exists in whale dentin as well as in the cementum but in very much smaller concentration.

Phosphotungstic acid precipitation: When fraction WD4 was dissolved in water and treated with phosphotungstic acid, a small precipitate was formed. This was not observed in the similar extract WC4 from cementum. This precipitate was collected, decomposed, and hydrolyzed with 6N HCl. The ultraviolet absorption spectrum in 0.1N HCl had $\lambda_{max.}$ 265 mμ and in 0.1N NaOH, $\lambda_{max.}$ 278-280 mμ. This result suggests a very small quantity of an

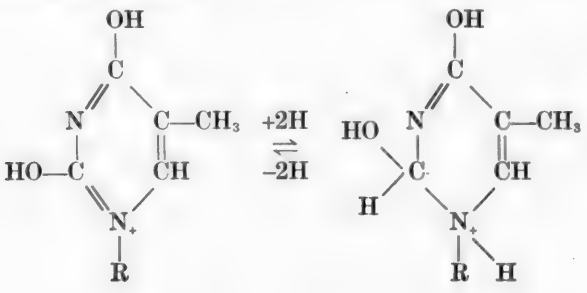
ether-soluble pyrimidine. Its forming a complex with phosphotungstic acid indicates the presence of an amino group; ether solubility, as in the case of thymine, may be due to a methyl group. These rather sketchy data suggest that a compound of the type of 5-methylcytosine might be present. This pyrimidine is rather a rare one, and further study would be of interest. This will have to be deferred until the return of the whaling fleet provides us with more material.

DISCUSSION

Ultraviolet and infrared spectroscopy provide strong evidence that whale cementum contains the pyrimidine thymine. One discrepancy in the evidence must be mentioned. The ultraviolet absorption spectra of compound T and thymine are identical with respect to maxima and minima and shape of curve. The value of $E^{1\%}_{1\,cm.}$ for compound T is, however, higher than that for thymine. Since the infrared spectra are in very close agreement, we feel justified in concluding that thymine does exist in whale cementum and is probably present to a much smaller extent in the dentin. We have little information concerning the nature of the parent compound S_2. The most likely substance would be a nucleic acid derivative of larger size than a pyrimidine, probably a nucleoside. It appears that thymine is found in combination only with desoxyribose and not with ribose.[10] According to Levene and Bass,[10] thymine desoxyriboside melts at 185° C. and will therefore, if present, be above its melting point during the treatment with alkaline ethylene glycol. It is unlikely that the desoxyriboside would remain unaltered by this treatment, even though its hydrolysis was not complete. We were unable to obtain any positive tests for pentose on any of our fractions.

Nitrogen analysis of S_2 indicates (on the basis of 2 nitrogen atoms) a molecular weight of about 220. Increase in weight on acetylation (on a basis of 2 potential hydroxyl groups) suggests a molecular weight of about 215. The molecular weight of thymine desoxyriboside is 242. It is possible, therefore, that the compound S_2 is derived from the desoxyriboside. It is almost certainly substituted in position 3 since such substitution is known to prevent the shift of maximum absorption to longer wave length in alkaline solution.

The fluorescence of our extracts is destroyed by reduction with sodium dithionite and can be restored by reoxidation. This suggests that the following type of system may be involved:



We feel justified, therefore, in suggesting that a nucleoprotein containing a desoxyribonucleic acid is a component of whale cementum and probably of the dentin. This is interesting since it is believed that desoxyribonucleic acids usually preponderate in the nuclear material, the ribonucleic acids being found in greatest quantity in the cytoplasm.[1] Neither cementum nor dentin can be considered to contain a large number of nuclei. It may be, of course, that we have given undue prominence to thymine since it happens to be extractable in ether. Other ether-insoluble pyrimidines may still be undetected in the main alkaline hydrolysate. This problem is being investigated.

It must be emphasized that the ether-soluble fraction accounts for only a very small proportion of the total fluorescence of our digest. We are not claiming that thymine is responsible for the whole fluorescence of whale cementum or dentin. It is, however, interesting to note that fluorescent photomicrographs reveal that the fluorescence of human dentin is concentrated in the dentinal tubules.[4] These tubules contain the dentinal fibrils or Tomes's fibers, which are considered to be cytoplasmic processes of the odontoblasts.[13] It has also been suggested that mitochondria are present in the Tomes's fibers.[14] It is possible therefore that the nucleoproteins exist within the Tomes's fiber and would at least contribute to the fluorescence. In an earlier study of human dentin,[6] many fluorescent fractions were obtained whose absorption spectra showed $\lambda_{max.}$ 250-270 mμ and $\lambda_{min.}$ 230-240 mμ. These are akin to the absorption spectra of many pyrimidines except that no shift to longer wave length in alkaline solution was noted. We now realize that this may be due to substitution in position 3 of the pyrimidine molecule. This human material, which was not soluble in ether, and, therefore, differs from that described in the present work, should repay reinvestigation.

Wislocki and Sognnaes[18] reported that basophilia present in the cytoplasm of odontoblasts and ameloblasts were attributable to ribonucleoprotein. More recently Johnson and Bevelander[9] have demonstrated by histochemical methods that both ribo- and desoxyribonucleic acids are components of the ameloblasts and odontoblasts of developing incisor teeth of pig embryos. They observed that the nucleic acids in these cells gradually appear as the cells differentiate and reach a high level which is maintained during the functional period.

SUMMARY

1. Thymine (2, 6-dioxy-5-methylpyrimidine) has been isolated from the cementum of the sperm whale.

2. A very similar pyrimidine has been detected in the dentin, but in much less quantity.

3. An ether-soluble basic pyrimidine has also been detected in the dentin.

4. It would seem likely that these pyrimidines are derived from nucleoproteins in the dental tissues of the sperm whale.

5. The nucleoproteins of the dentinal tubules may contribute to the fluorescence of dentin.

We wish to thank Professor R. A. Morton, F.R.S., for his interest in this work. Thanks are also due to Mr. M. Carruthers for assistance in infrared spectroscopy, and to Messrs. Henry Tyrer & Co., Ltd., of Liverpool for the gift of sperm whale teeth. One of us (A. G. L.) is grateful to the Medical Research Council for the receipt of a full-time studentship.

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PHOTOELASTICITY AS A RESEARCH TECHNIQUE FOR ANALYZING STRESSES IN DENTAL STRUCTURES

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THE primary purpose of the masticatory process is to apply forces to the nutritional substances which are taken into the mouth, thereby causing a reduction in their size. The mechanism for this action includes the medium of tooth structure which not only applies these forces directly to the food masses, but is itself subject to the effects of these forces.

In general, the effects of forces applied to a body are manifest in the development of internal stresses which are distributed in accordance with the direction of the applied forces, the manner in which the body is supported, and the shape of the body. Furthermore, these stresses are accompanied by internal deformations or strains within the body, and if their magnitudes are sufficiently large, permanent deformation and, ultimately, failure will ensue. Tooth structure and dental restorations are not exceptions to these rules. The structural design of normal tooth structures is such that they withstand the effects of masticatory forces. However, when tooth structure is removed and replaced by a dental restoration, the normal internal stress distribution is altered and the new stress situation will depend on the design of the cavity preparation. Furthermore, the shape of the restoration, which is also determined by the cavity preparation, is of considerable importance as to whether the restorative material will undergo permanent deformation or fracture.

In general, then, an evaluation of the internal stresses in both dental restorations and tooth structures should yield valuable criteria for successful design.

The consideration of stresses in dental structures is not a new concept. There has been considerable reflection concerning the design of dental restorations so as to avoid failure caused by internal stresses. Most of these efforts have resulted in generalities deduced from the theory of elasticity as applied to uniformly shaped structures and, therefore, leave much to be desired when confronted by the irregular shapes of dental structures. Empirical rules or criteria have developed over the years on the basis of clinical evidence and these have served to avoid the deleterious effects of internal stress magnitudes. However, since both the design and size of dental restorations are extremely limited by the biologic aspects of tooth structure, an exacting analysis is the only solution to the determination of an optimum structural configuration.

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When consideration is given to the irregular shapes of dental restorations, an experimental method of stress analysis is indicated since mathematical theories can be applied only to certain regular shapes. Photoelasticity is such an experimental method and it is the purpose of this paper to describe this method and its application to dental restorative design. The work of Noonan,[1] Castro,[2] and King[3*] is representative of the first concrete attempts to utilize this method in the solution of dental problems with regard to structural design.

A 3-dimensional analysis should be considered since most dental structures vary in shape and loading characteristics in 3 dimensions. However, for purposes of simplicity and convenience, the following discussion is for a 2-dimensional analysis, from which a 3-dimensional approach can be derived, where indicated. The engineering literature is quite thorough in its treatment of photoelasticity, and for more complete information, which is beyond the scope of this paper, reference is made to Frocht.[4, 5]

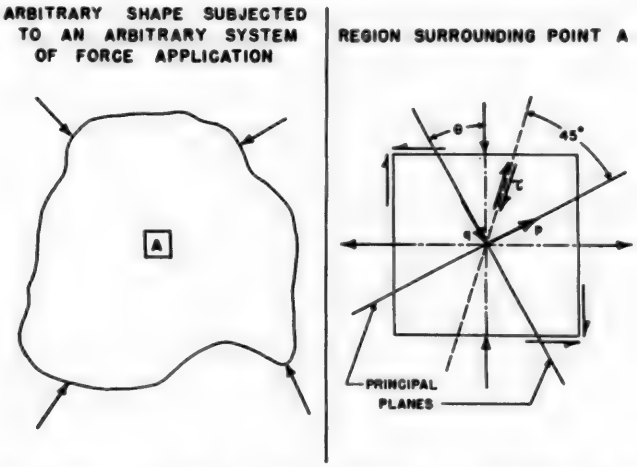


Fig. 1.—Stresses acting at a point in a body, illustrating the orientation of the principal or maximum stresses p and q as well as the maximum shear stress, τ.

PRINCIPAL STRESSES

The general procedure in structural design is to evaluate the maximum stresses which exist in a structure and relate these to the inherent strength properties of the materials which compose the structure. As a consequence, the first step in analysis would be to determine the maximum stresses. These stresses are illustrated in Fig. 1 for a 2-dimensional analysis. The sketch on the left-hand side is a schematic representation of a body of arbitrary shape subjected to an arbitrary system of force application (dental structures are simply specific cases of this general system). To investigate the internal stresses at any point in this body, that is, point A, it is convenient to magnify a small region surrounding this point. This situation is shown on the right-hand side

*Since preparation of this paper two additional references to this subject have appeared: Haskins, R. C., Haack, D. C., and Ireland, R. L., Study of Stress Pattern Variations in Class II Cavity Restorations as a Result of Different Cavity Designs, *J. D. Res.* **33**: 757, 1954, and Watton, C. B., and Seven, M. M.: A Preliminary Report of Photoelastic Tests of Strain Patterns Within Jacket Crowns, *J.A.D.A.* **50**: 44, 1955.

of Fig. 1. The stresses which are shown acting on the sides of the square element are those produced by the external force system and represent a general system of plane stress, that is, compressive, tensile, and shear type stresses are shown acting on this square element. The magnitude of stresses on an arbitrary plane passed through the element can be determined by resolving the stresses acting on the external sides of the element. As a further condition, if this plane is specifically oriented such that no shearing stresses are present, then the normal (perpendicular) stress to this plane is the algebraic maximum stress which exists at point A (corresponds in most cases to the largest tensile stress). Similarly, another plane, perpendicular to the aforementioned plane, exists upon which no shearing stresses act and the normal stress is the algebraic minimum stress at point A (corresponds in most cases to the largest compressive stress). These stresses are shown in Fig. 1 and their normal connotation is that p is the algebraic maximum stress and q is the algebraic minimum stress. The planes upon which these stresses act are known as principal planes. Furthermore, on a plane at 45 degrees to these principal planes, the acting shear stress τ is a maximum and the following relationship is valid.

$$\tau_{max} = \frac{p - q}{2}$$

Although considerable work has been done in the field of engineering on the phenomenon of plastic deformation and fracture of solid structures, the actual relationship between the physical properties which describe the failure processes and the maximum internal stresses has not been defined exactly; this relationship being strongly dependent upon the type of material used and the way in which stress is applied to the material. Despite this inadequacy, knowledge of the maximum stresses serves as a firm basis for the analysis of structural designs.

PHOTOELASTIC METHOD

Photoelasticity is an experimental method for evaluating the stresses responsible for failure of a structure. The general procedure is to construct a model of the structure to be investigated from what is known as a photoelastic material. The direction and magnitude of the applied forces on the model, the way in which the model is supported, and the shape of the model must be similar to the conditions of the actual structure. The internal stresses in the model will then be similar to those existing in the actual structure regardless of material (assuming that the elastic limit has not been exceeded and that the original structure is a homogeneous isotropic material). This point establishes its validity from the absence of material physical constants from the stress distribution relationship of 2-dimensional systems. Experimental verification of this point also exists.

The temporary double refraction under stress of certain transparent isotropic materials (photoelastic materials) is utilized for photoelastic analysis. This property is such that an incident ray of light will be resolved into two rays which travel along the principal planes of the material at the point of incidence, the two rays traveling at different velocities and one emerging retarded with respect to the other. The amount of retardation is directly proportional to the difference between the principal stresses.

The photoelastic polariscope is used to measure the retardation, thereby measuring the difference between the principal stresses. The mechanism of measurement utilizes the property of light-wave interference (Fig. 2). The wave theory of light is used to explain this phenomenon. For simplicity, a monochromatic light source is shown (Fig. 2) which transmits light waves in all directions to the polarizer. The polarizer has the property of passing only those components of the incident light waves which are parallel to the polarizing axis. Consequently, plane polarized light is produced which impinges upon the stressed model made of a material exhibiting temporary double refraction under stress. Only those components of the incident wave which are parallel to the principal planes of the model at the point of incidence pass through, and these components are retarded in accordance with the degree of stress existing at the point in question. The two light waves then fall on the analyzer, which is physically the same as the polarizer except that its axis has been rotated 90 degrees. This would produce a dark field if no model were present. The

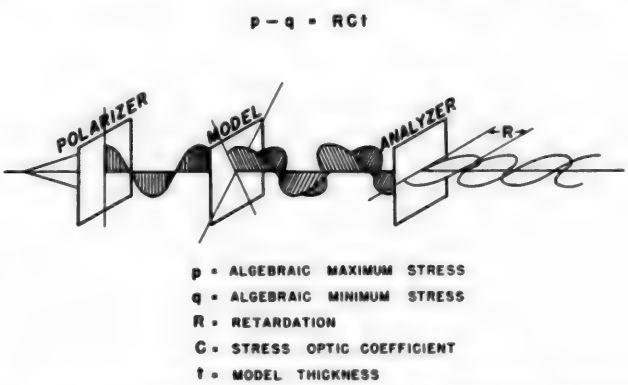


Fig. 2.—Schematic sketch of the operation of a photoelastic polariscope.

components of the incident light waves which are parallel to the polarizing axis of the analyzer are then passed through, and since they lie in the same plane, interference phenomena can occur. When the retardation is such that the paths oppose each other, a dark spot will appear, and when in-between conditions occur, some light will get through. Adjacent dark and light spots will then form alternate dark and light lines or fringes which are directly proportional to the principal stress difference existing in the model. The specific relationship of stress and retardation is given as:

$$p - q = aR.$$

Where a is a constant depending on the material from which the model was made and the thickness of the model, R is the retardation experienced by the light beam.

If a white light source is used, the light wave is made up of a number of waves having different frequencies. When one of the component waves is eradicated by interference, its complementary color remains and the entire

effect is a spectrum of complementary colors. For example, when violet is extinguished, its complementary color of yellow is visible. Each color, then, represents a different degree of principal stress difference. In the field of photoelasticity, colored fringes were initially used for stress determination. However, the dark and light bands which result from the usage of a monochromatic source are employed today, almost exclusively, because of their superior quantitative accuracy. This is due mainly to the fading of the colored bands in the vicinity of high stress concentrations, caused by an overlapping of complementary colors.

The colored patterns, however, do serve to yield additional information necessary for the complete analysis of stress. This information is the direction of the principal stresses. Dark lines can be observed superimposed on the colored stress patterns. These occur whenever the plane of the incident polarized light is coincident with the principal planes of the point in question and as a consequence the light beam is not resolved and passes directly through the model. The analyzer, being perpendicular, nullifies this light, and dark spots appear, the locus of which are dark lines known as isoclinic lines. These lines, then, in representing the direction of the principal planes, are indicative of the directions of the principal stresses. By rotating the plane of the incident light beam, all of the isoclinic lines can be determined.

In order to evaluate the principal stresses individually, additional information is required. This individual evaluation is necessary to predict failure in accordance with accepted theories. Although there are several procedures available, based on the photoelastic data, an experimental method exists which enables the determination of the sum of the principal stress $(p + q)$. The merit of this auxiliary method is that it is a separate source of principal stress information, independent of the photoelastic data. It is based on the measurement of the lateral deformation of the model under stress and the relationship which exists is:

$$p + q = SK.$$

Where K is a constant depending on the physical constants of the photoelastic materials being used and the thickness of the Model, S is the lateral deformation of the model under stress.

Having determined the value of $p - q$ by the photoelastic method and the value of $p + q$ by the lateral deformation method, the individual value of p and q can be easily evaluated. This information, together with the direction of the principal stresses as determined by the isoclinic lines, is sufficient for the complete analysis of the significant stresses developed in dental structures as a result of applied occlusal forces.

APPARATUS AND TEST PROCEDURE

The photoelastic polariscope which has been designed and assembled at the Physical Research Laboratory of the University of Michigan School of Dentistry uses white light or monochromatic light lamps interchangeably as a light source, an aperture approximating a point source at the focal length of the lens system, a filter used with monochromatic light source, a condensing lens

to produce parallel rays in the field of the model, a quarter-wave plate to produce circular polarized light which eliminates the isoclinic lines, a beam type of loading mechanism with a water tank at the end, by means of which the load is adjusted, catalin 61-893 model material, a second quarter-wave plate to resolve emerging light waves, a polaroid analyzer to resolve light waves into the same plane, a collecting lens to converge parallel field rays, and a camera to record patterns.

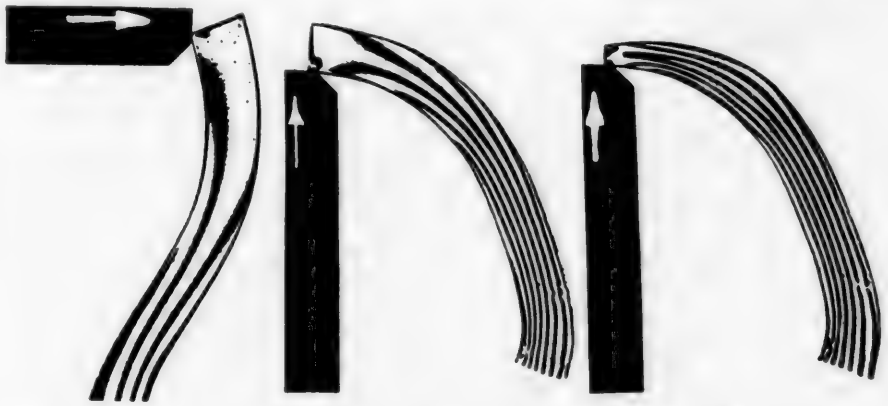
A lateral extensometer is used for measuring lateral deformation which yields the subsequent (p + q) value. This device, designed to incorporate the Tuckerman Strain Gage, was patterned after Peterson and Wahl's[6] extensometer. It can record a dimensional change of less than 1 micron.

Fig. 3.—Stress distribution in a buccolingual section of a molar tooth subjected to an occlusal load for (*A*) a normal tooth, (*B*) one restored with an amalgam restoration, and (*C*) one restored with a gold inlay preparation.

The procedure of test is as follows and refers specifically to the handling of Catalin 61-893, a photoelastically sensitive plastic, which is available in roughly ground plates: the shape of the structure to be analyzed is drawn on a piece of Catalin, which is then cut moderately close to this outline; the model blank is then ground to a uniform thickness on successively finer polishing papers and finish-polished on a metallographic polishing wheel; the model is then trimmed to the final outline. Since the stress-retardation relationship is dependent upon the model thickness, it is imperative that this factor remain constant. For this reason, the procedure given here is followed since the polishing operation tends to round the edges of the model and, therefore, must be done before the final outline is cut. A masking tape can be used to protect the polish of the model when the final outline is cut. The model is then placed in the loading mechanism

with a supporting structure and load application designed to approximate, as closely as possible, the actual conditions of the structure being investigated. The pattern is then observed and photographed.

In interpreting the stress pattern it is important to realize that each dark or light band is a locus of points representing constant shear stress or constant principal stress difference. In addition, the bands which appear will be of different orders such that the first order band will represent a given value of stress while the second order band will represent twice the value of stress as the first order band, and so on. There are ways of determining what order band exists at a point in question but the simplest method is to observe the pattern formation while loading and the order of bands can thus be easily determined.



A. *B.* *C.*

Fig. 4.—Stress distribution in a partial denture wrought clasp under the action of (*A*) masticatory loads, (*B*) the force necessary for removal, and (*C*) the force necessary for removal but using a tapered clasp.

APPLICATIONS OF PHOTOELASTICITY IN DENTISTRY

A number of examples were prepared to indicate the types of dental problems for which this method is especially suited. They were arbitrarily chosen and are representative rather than inclusive examples.

In Fig. 3 is shown a buccolingual section of a mandibular right permanent first molar subjected to an occlusal load. The support for the tooth was made of aluminum and shaped to approximate the bone structure. The tooth section was sealed to the aluminum mold with impression compound. The load was applied through a round pin to approximate the opposing cusp. These pictures show the stress distribution of a normal tooth section (*A*), a section restored with an amalgam type restoration (*B*), and a section restored with an inlay type restoration (*C*). If the bands are counted from the tips of the cusps which are free from stress, we find a concentration of 4 light bands at the pulp horn on the left for the normal tooth section. For the amalgam restoration, the concentration is about 5½ whereas the inlay restoration is about 7 bands. Since these bands are directly proportional to the shear stress, it can be con-

cluded that the placement of the restoration has significantly increased the stress concentration at the point under consideration and furthermore that this increase is dependent upon the design of the cavity preparation.

In Fig. 4, a partial denture-wrought clasp used on a bilateral type of restoration is analyzed. The buccal view of the clasp is shown (*A*) with the force applied as a result of resorption in the saddle area and a consequent tipping of the restoration under vertical occlusal loads. This force was determined by assuming the amount of deflection which would occur at the end of the clasp under the conditions previously stated. The occlusal view of the clasp (*B*) is shown with the force necessary to remove the restoration. This was determined by the amount of deflection required to allow the clasp to escape the contour of the abutment tooth. The same view is shown (*C*) with the exception of a tapering of the terminal third of the clasp. This particular case is presented because of the high incidence of clasp failures which occur in practice. The preliminary conclusions which can be drawn from these illustrations are that the stresses induced in the clasp are greater upon removal than under the action of masticatory loads and tapering the terminal third of the clasp slightly reduces the stresses induced in the clasp but in addition reduces the retention. It would appear that a relatively large cross-sectioned clasp which would reduce the stress at the point of attachment would prevent failure, and if a taper were to be employed, the retention could be brought to within desirable limits.

SUMMARY

It has been indicated that the analysis of stresses in dental structures would establish valuable criteria for the design of cavity preparations and dental restorations. The experimental method of photoelasticity was described in theory and practice, and two examples were presented to show the application of this method to the analysis of stress in dental structures. This method of analysis was shown to be particularly applicable to dental problems because of the irregular shapes of dental structures.

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MEASUREMENT OF THE ELECTRIC CONDUCTIVITY OF DENTAL CEMENT

IV. Extracted Human Teeth; In Vivo Tests; Summary

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THE presence of small galvanic currents between similar or dissimilar metals in the oral cavity has been well established.[1-3] It has been suggested that such currents are one possible cause of oral lesions,[1] that they produce corrosion products which penetrate into the dentin and produce discoloration,[4, 5] and that they may cause postoperative pain.[6] For these reasons, cement bases beneath gold and amalgam restorations have long been advocated to provide an insulating barrier against both thermal and electric shock to the pulp. Although Schriever and Diamond[1] carefully determined the magnitude of these currents and other investigators have studied the general problem, the actual insulating properties of these materials and the electric conductivity of dental cements have not been determined. Whether the cement base aids in reducing the galvanic current has been unknown.

We have investigated basic values for the electric conductivity of dental cements and their clinical effectiveness in insulating the tooth against electric energy. Experimental procedures, instrumentation, and the mathematical calculations for accurately measuring this conductivity were developed.[7]

It was found that zinc phosphate cement, zinc oxide–eugenol, and acrylic resin were all effective insulators when the cement was sealed against moisture (approximately 1,000 megohms at the end of 800 hours). However, when the zinc phosphate cement margins were exposed to distilled water or normal saline solution, the resistance was greatly reduced, dropping to a value which might indicate that its insulating ability, clinically, is questionable.[8] These findings were corroborated with several types of experimental cells, including one group which simulated the cemented gold crown. Resin cement and zinc oxide–eugenol continued to have high insulating ability even when the margin was exposed to moisture.[9]

The investigation up to this point had clearly indicated a marked difference in the electric conductivity of zinc phosphate cement when exposed to moisture, as compared to when it was maintained dry. Likewise, the continued good insulating characteristics of both resin and zinc oxide–eugenol, even under wet conditions, were noted. The exact amount of moisture to which the cement is exposed clinically is, of course, unknown. Undoubtedly, some moisture is present in the dentin itself, and moisture probably enters along the walls of

This investigation was supported in part by a grant from the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, Contract No. DA-49-007-MD-391. A portion of this paper was read to the Dental Materials Group at the 32nd Annual Meeting of the International Association for Dental Research, French Lick, Ind., March 21, 1954 (J. D. Res. 33: 511, 1954).

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the preparation due to marginal leakage.[10-12] In order to accentuate the effect of moisture, rather exaggerated conditions had been used. Since the answer to the effectiveness of these materials in insulating the pulp against electric energy depends upon the amount of moisture to which they are subjected, it was felt that an additional series of tests, both in vitro and in vivo, should be made.

IN VITRO—EXTRACTED HUMAN TEETH

Previously, all the results had been obtained from work on experimental cells which did not involve teeth. These cells had been designed for minimizing test variables in order to obtain basic values. A closer approach to the actual clinical condition could be made if extracted human teeth were used. Consequently, a series of experiments using dissociated human teeth, noncarious molars stored in tap water, were initiated.

A circular occlusal cavity was prepared in each tooth. The cavities were undercut and were approximately 0.25 inch in diameter at the pulpal floor. The apical two-thirds of the root had been removed so that the pulp cavity and cervical portion of the pulp canal, where present, were widely exposed. The teeth were then mounted in a block of hydrocal, 4 or 5 teeth to a block, with the crowns exposed above the stone as seen in Fig. 1. A strip of copper gauze was embedded in the hydrocal beneath the teeth to afford one electric connection, and the other connection was made to the amalgam restoration. The embedded teeth were stored in 1 per cent saline solution. The current path was thus through the saline solution–saturated stone block to the copper gauze. The resistance of the stone (0.005 megohm) was accounted for in the mathematical calculations.

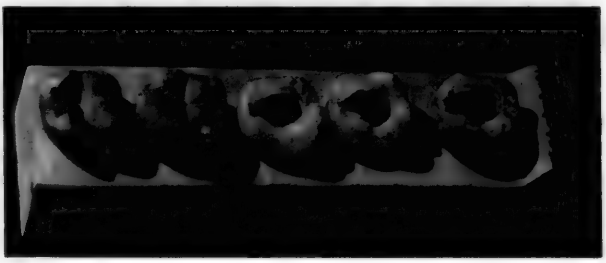

Fig. 1.—Cells in extracted teeth showing both cavity preparation and amalgam restoration.

Series I.—This series involved 14 teeth divided into 4 groups. All prepared cavities were dried with alcohol and air. The first group had no cement on either the walls or the floor of the preparation. The second group had zinc phosphate cement only on the floor. The third group had acrylic cement on the walls and nothing on the floor. The last group had acrylic cement on the walls and zinc phosphate on the floor; the resin was used here to insulate the side walls from the metal restorations. The preparations were restored with amalgam. This series would thus afford data relative to: (1) basic values for dentin (2) the effect of the zinc phosphate base; and (3) the possibility of an additional path of conduction of electric energy through the side walls of the cavity preparation.

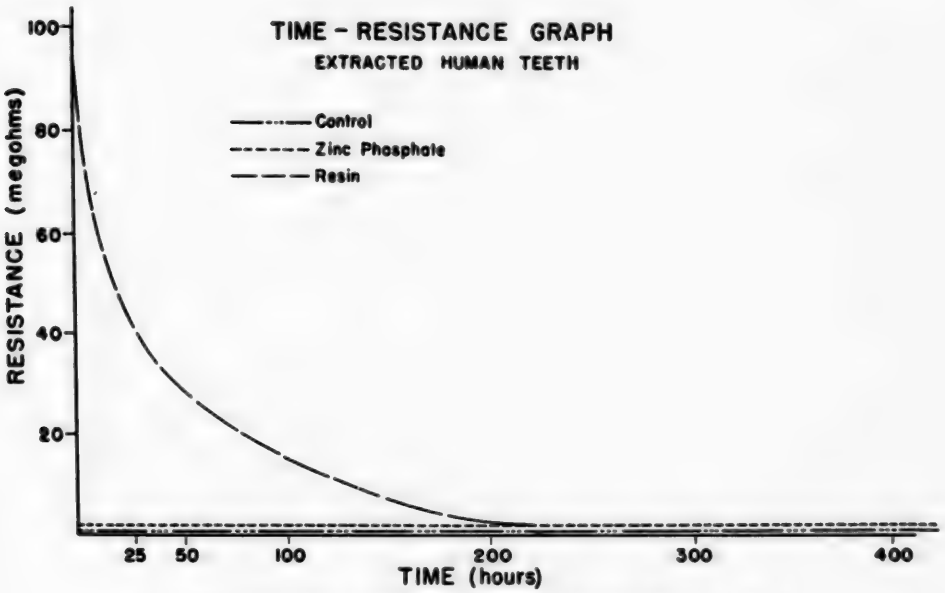

Fig. 2.—Resistance measurements taken on cells shown in Fig. 1.

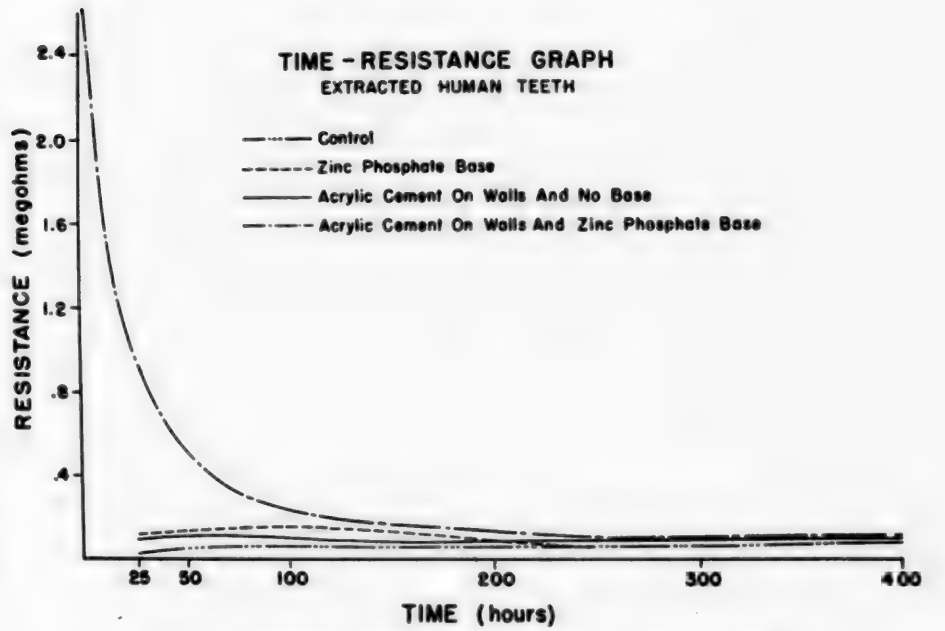

Fig. 3.—Resistance measurements taken on cells shown in Fig. 1.

The supporting block was covered with saline solution with the crowns of the teeth exposed to air. The exposed portion of the tooth was maintained at 100 per cent humidity when readings were not being taken. Circuit B was employed for measuring the current, and the resistance of the tooth was calculated.[7] All readings were taken at room temperature. Before the data had been recorded, the crowns were thoroughly dried in order to block the current path over the crown surface from the metal filling to the hydrocal.

The mean data are plotted in Fig. 2. The addition of the cement base did not effectively increase the resistance of the dentin. In all cases, the resistance of the cementing material is far below the basic units which were obtained for dry specimens.[7] It is comparable to the values previously found for zinc phosphate cement under wet conditions.[8]

In the specimens where the walls were lined with acrylic cement and the zinc phosphate cement base was used, the initial values of resistance were relatively higher. However, the rapidly decreasing resistance indicates poor insulating ability, and eventually, values were approximately the same for all 4 conditions. When used with the base, the acrylic cement on the walls seemed to contribute only to the early values of resistance. This would indicate that the walls themselves eventually provide a path for conduction of electrical energy. As time progressed, moisture evidently neutralized the initial effectiveness of the acrylic cement on the walls.

Series II.—In this experiment the teeth used in Series I were again employed. The amalgam and cement were removed from the teeth, exposing the dentin wall and floor. One tooth was restored with amalgam only and thus served as the control. The other teeth were restored with amalgam using: (1) a thick (approximately 1 mm.) zinc phosphate cement layer on both walls and floor; and (2) a resin cement on walls and floor. Such conditions simulated the clinical use of these materials both as a base and as a luting material for inlays. It also provided data relative to increased thickness of the cement layer.

The results can be seen in Fig. 3, where the mean resistance is plotted against time. The teeth which had zinc phosphate cement on the walls and floor had resistance values slightly higher than the control, the average value being approximately 1 megohm. This resistance was higher than the values obtained from the previous work with wet cells. Since it was thought that the original cells had an exaggerated moisture condition, possibly the degree of moisture present accounts for the difference. However, whether or not these restorations in extracted teeth simulate the in vivo conditions is also not known.

The values of resistance for resin cement were higher. Initial measurements showed adequate resistance. Previous work has indicated that resin cement is a satisfactory insulator both wet and dry, whenever the margins are not short-circuited. In other words, if the marginal seal is maintained, insulation does exist. In this experiment, resistance for resin cement was high initially but it decreased with time until the value was approximately 2.5 megohms. Apparently, moisture did penetrate the margins and the seal was partially broken. The actual degree to which the resistance of the cement was being neutralized by the parallel leakage path is not known. The crowns of the teeth had been

TABLE I

TIME OF READING AFTER CELL ASSEMBLY (HOURS)	CELL NUMBER AND RESISTANCE IN MEGOHMS													
	METHYL METHACRYLATE		POLYSTYRENE		2 LAYERS REPELAC			1 LAYER REPELAC		4 LAYERS COPALITE			2 LAYERS COPALITE	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
2	0.248	0.063	0.187	0.960	0.237	0.355	0.300	0.175	0.187	0.053	0.063	0.084	0.040	0.063
8	0.287	0.053	0.130	0.755	0.287	0.355	0.160	0.175	0.237	0.063	0.063	0.083	0.053	0.084
24	0.187	0.063	0.074	0.510	0.237	0.395	0.224	0.175	0.287	0.033	0.053	0.053	0.033	0.033
168	0.118	0.040	0.040	0.187	0.200	0.237	0.187	0.118	0.130	0.040	0.040	0.074	0.033	0.033
240	0.084	0.053	0.053	0.063	0.063	0.063	0.074	0.053	0.074	0.063	0.040	0.033	0.033	0.040
336	0.063	0.040	0.053	0.040	0.040	0.084	0.040	0.053	0.074	0.040	0.033	0.033	0.033	0.022

dried thoroughly to prevent any short-circuiting effect at the margin. It would seem, then, that in this particular series of tests both zinc phosphate and resin cement, when lining the entire cavity, did contribute somewhat to the insulation of the metallic restoration. The next step would be to determine whether the in vivo conditions correspond to these in vitro conditions, both in respect to the degree of moisture present and the conductivity of live dentin as compared to dead dentin.

Series III.—After learning that lining the entire cavity did slightly increase the resistance, further experiments using commercial cavity liners, as well as some experimental ones, were conducted. In this series, the teeth used previously were again employed in order to eliminate differences in individual teeth. The cavity walls and floor were coated with the following agents: experimental liners of methyl methacrylate and polystyrene and commercial liners Repelac and Copalite. No cement bases were used, and amalgam was packed directly against the liner.

Initially (Table I) all 4 materials had little insulating ability, polystyrene being the best. Copalite, even when applied in several layers, was quite ineffective. At the end of the test period all materials had resistance values which were comparable to those obtained in the control teeth. The use of these materials as liners in a cavity preparation does not increase the insulation to electric energy.

Series IV.—A group of 5 cells was constructed in which silver nitrate was reduced with eugenol in the cavity preparation before the restoration was inserted. The results showed that the precipitation of silver from silver nitrate upon the cavity walls had no appreciable effect upon the conduction of current from a metal filling through the tooth.

IN VIVO TESTS

The molar teeth of 2 dogs were used in this experiment. The preparations employed are shown in Fig. 4. The size of the preparation was approximately that of a No. 560 crosscut fissure bur. The position of the preparation was carefully located to avoid a pulp exposure. In the first dog, a cell of 2 amalgam plugs separated by a dentin wall, about 1.7 mm. thick, was prepared in one tooth (A). In another tooth, a cell of 2 gold plugs, separated by zinc phosphate cement, was formed (*B*). In a second dog, 2 additional cells were constructed. In *C*, the 2 amalgam plugs were separated by a 2.0 mm. plug of zinc phosphate cement and in *D* the 2 amalgam plugs were separated by a 1.9 mm. plug of resin cement. A special pair of probes were designed to be used for taking the readings. They provided adequate insulation in order to prevent any appreciable error due to capacitance effect from the hands. The tooth was thoroughly dried and resistance measurements were made utilizing the B circuit system.[7] Measurements were made over a period of two and one-half months.

The magnitude of resistance varied from 0 to 1.06 megohms, as shown in Table II. There was little difference between the 4 cells, all materials having approximately the same low values of resistance. It was interesting to note that the dentin (Cell A) produced approximately the same resistance as the other

TABLE II
RESISTANCE MEASUREMENTS IN VIVO (DOGS)

TIME OF READING AFTER INSERTION	RESISTANCE OF CELLS IN MEGOHMS			
	A	B	C	D
1 hr.	0.79	0.0	0.0	0.0
1 wk.	0.18	0.34	0.31	0.29
2 wk.	0.27	0.29	0.29	0.49
3 wk.	0.37	0.32	0.51	0.27
5 wk.	0.41	1.06	0.20	0.20
7 wk.	0.29	0.87	0.59	0.31
Extracted and measured	0.26	2.00	0.55	0.22
Stored in saline solution 2 days	1.66	7.37	1.72	1.30
Pulp chamber opened to saline solution	0.49	5.77	0.66	0.26
24 hr. in saline solution	0.41	3.16	0.21	0.17
Dried and stored in air—20 hr.	270	314	360	677
Saline solution injected into pulp chamber	0.13	10.8	0.18	0.08
Dried and stored in air 138 hr.	>1,000	>1,000	>1,000	>1,000
Saline solution injected into pulp chamber	0.19	0.07	1.47	0.06

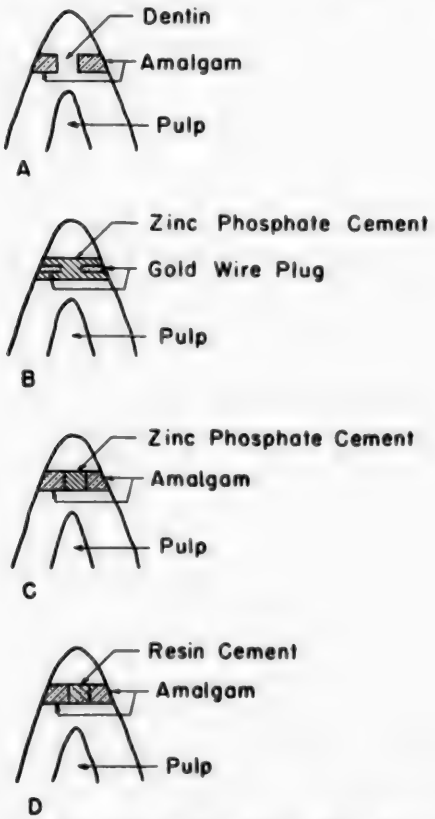

Fig. 4.—In vivo cells showing various types measured.

materials. Previous in vitro tests had shown that resin, under both wet and dry conditions, was a good insulator; however, in these preparations the resistance of the resin was apparently short-circuited. It is possible then that the

dentin itself may act as the short circuit in all the teeth. The average value of resistance for the dentin cell was 0.41 megohms, the resin cell was 0.31 megohms, and the zinc phosphate cell, 0.33 megohms. These values are of similar magnitude and indicate that the electric path could be the same in all three cases. The in vivo tooth is apparently a better electric conductor than the dry cementing materials.

These teeth were extracted and stored in normal saline solution immediately and the measurements continued. The resistance at the end of 2 days increased. The pulp chamber was then opened to the saline solution and the resistance decreased. The teeth were dried and measured over a period of 20 hours. The resistance increased to values between 270 and 677 megohms. Saline solution was then injected into the pulp chamber and the resistance immediately fell. This procedure was repeated several times and results were repetitious. Under dry conditions, high resistance was obtained, but low values resulted when saline solution was injected into the pulp chamber. The effect of moisture was dramatically demonstrated in this experiment. The short-circuiting of these materials when placed in the tooth prevents maximum insulation.

DISCUSSION

There has been no basic information published on the electric conductivity of dental cements and the influence played by such variables as thickness, contact area, aging, and so on. In a series of papers[7-9] from this laboratory, there was presented the development of the instrumentation, circuit design, and cell construction which has made possible accurate determination of the electric conductivity and thus the insulating ability of these materials. The question arises concerning what, from a dental standpoint, might be termed "adequate insulation." It is difficult to estimate the actual value of resistance which would be effective in the clinical restoration. An arbitrary magnitude of resistance which would reduce the amount of current flow to one twentieth of its original value was accepted by the authors as being adequate. Consequently, any material which would provide such a resistance was designated as having effective insulating ability.

When maintained in a dry condition, all the materials tested, except calcium hydroxide, were adequate insulators. Both resin and zinc oxide–eugenol paste also effectively insulated when exposed to saline solution. Calcium hydroxide and zinc phosphate cement were poor insulators under these wet conditions.

In these original cells it became necessary to determine whether the loss in insulation was due to the current passing through the wet material or actually by-passing the cement. Leakage at the margins was an extremely important problem. Early work showed that when the marginal seal was maintained, resin cement and zinc oxide–eugenol paste were effective insulators. Yet, even in these cells, if the marginal seal was broken, then conduction resulted. Naturally, the practical significance of this finding is whether an effective seal can be maintained under clinical conditions. This seems unlikely since none of the restorative materials used today forms a bond with the dentinal wall adequate to prevent the penetration of moisture.[10-14]

In order to simulate clinical conditions more closely and verify the former observations, the series with extracted teeth were run. The previous results were confirmed. It made little difference what type of cavity liner or base was used because if moisture was present the insulating ability was greatly lowered. Moisture apparently reaches the cement through both the dentinal wall and the margin. In the case of zinc phosphate cement there is an actual conduction through the wet material, while with resin cement the conducting path is around the margin. The work with dogs also substantiates the fact that even though a material basically may be a good electric insulator, both wet and dry, clinically its effectiveness is greatly reduced. It is of little benefit, electrically, to place a good insulator in the cavity preparation if it is short-circuited by the live dentin and by marginal leakage. The possibility of eliminating these sources of moisture, with present materials and techniques, appears improbable.

Schriever and Diamond[1] made no mention of cement bases. The results of our work make it apparent that little difference would have been noted in their measurement if the resistance of the cement base had been considered in their calculation. The work with dogs shows that the cement does not increase the effective resistance to current flow.

Postoperative pain which is associated with galvanism is a disturbing clinical problem. Assuming that the pain is caused by a certain minimum magnitude of current flow, the addition of any cement, regardless of thickness, area involved, composition, and so on, would not reduce the magnitude of that current. Likewise, Schriever and Diamond showed no correlation between the age of the restoration and the existing potentials or the current that flowed. Since these currents do exist in old as well as new restorations and since the cement base is not an effective insulator, it is more likely that the physiologic condition of the tooth is the primary factor responsible for the pain that results from this current flow. Once the tooth has responded from the injury of cavity preparation and returned to a more normal physiologic condition, the same magnitude of current flow may produce no response.

Apparently little can be done, practically, such as increasing the thickness or the area or altering the type of cementing material to improve the insulation against electric energy. The metallic restoration, for all practical purposes, cannot be insulated from the tooth in the mouth except by the application of extraoral varnishes which temporarily isolate the tooth from saliva. From the electric standpoint, only nonconducting restorative materials would eliminate completely the problem of pain-producing currents.

SUMMARY

Basic values for the electric conductivity of dental cementing materials have been determined in previous publications.[7-9] Data reported in this paper indicate that under in vivo conditions, moisture, either by penetrating through the material or around the margins, prevents insulation of the metallic restoration from the tooth. Cement bases, per se, contribute little or nothing to this insulation. It is very important that the results of this work be interpreted only with respect to electric conduction.

We wish to acknowledge the contributions of Mr. Robert Johnson for assistance in the in vivo tests, and of Mr. Richard Scott for photography throughout this investigation.

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A SIMPLE METHOD OF INCREASING THE ADHESION OF ACRYLIC FILLING MATERIALS TO ENAMEL SURFACES

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ONE of the major shortcomings of the acrylics and other filling materials is their lack of adhesion to tooth structure.[1-4] A filling material capable of forming strong bonds to tooth structures would offer many advantages over present ones. With such a material, there would be no need for retention and resistance form in cavity preparation, and effective sealing of pits, fissures, and beginning carious lesions could be realized.

In our attempts to obtain bonding between filling materials and tooth structure, several possibilities are being explored. These include (1) the development of new resin materials which have adhesive properties; (2) modification of present materials to make them adhesive; (3) the use of coatings as adhesive interface materials between filling and tooth; and (4) the alteration of the tooth surface by chemical treatment to produce a new surface to which present materials might adhere.

This last approach is the subject of this paper, but since it concerns itself only with treatment of intact enamel surfaces, it has only limited application to the broader problems of restorative dentistry.

In industry, phosphoric acid and preparations containing it have been used to treat metal surfaces to obtain better adhesion of paint and resin coatings.[5] Although the increased adhesion is believed to be due primarily to the removal of surface and other contaminants, the conversion of the oxides or the surface of the metal itself to phosphates or the adsorption of phosphate groups on the metal surface may contribute to the effect. Since the enamel surface has probably reacted with various ions, saliva, and so on, to which it has been exposed for long periods of time, and its tiny imperfections filled in by a variety of adventitious materials, the composition of the superficial surface may be quite different than the underlying enamel.[6] As a result, any receptivity to adhesion which the original tooth structure may have had for acrylic materials may have been lost. It was felt that perhaps an acid treatment of the enamel surface might render it more receptive to adhesion in the same manner as it does for metals.

EXPERIMENTAL

Two methods were used for treating the enamel surfaces. The first involved the use of a 50 per cent dilution of a commercial phosphomolybdate

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reagent containing sodium tungstate (Folin-Wü) in conjunction with a 10 per cent oxalic acid solution; the second, the use of an 85 per cent phosphoric acid solution.

By using the phosphomolybdate–oxalic acid treatment, we hoped to produce a surface chemically different from that existing normally in enamel. It was felt that the phosphoric acid–molybdate portion of the reagent would remove the superficial layers of enamel, and the tungstate group might link up with available organic material of the enamel. To further chemically change the enamel surface, it was treated with oxalic acid in the hope of incorporating oxalate groups on it, either by exchange or precipitation reaction.

The use of phosphoric acid alone was based on the thought that perhaps a simple decalcification resulting in removal of superficial structure was all that was necessary to produce a surface more receptive to adhesion. If this were so, it would eliminate the procedures used in the first method.

A qualitative comparison of adhesion was obtained by periodically testing the resistance to removal by thumbnail pressure of acrylic drops placed on treated and untreated enamel and dentin surfaces of extracted teeth that had been stored in water prior to and after affixing of the acrylic resin. Positive evidence of increased adhesion was obtained on the treated surfaces. Because the laboratory results were encouraging, it was decided to test the effect of surface treatments intraorally. Since previous experiments had shown that acrylic drops placed on untreated enamel surfaces of teeth in the mouth rapidly separated from them, it was felt that the duration of adhesion provided a good basis for determining the effects of surface treatments on adhesion.

The labial enamel surfaces of upper and lower incisors and occasionally premolars and molars of volunteer subjects were used. Paired experimental and control teeth were pumice-cleaned and alcohol-washed just before treatment.

The first method of treatment consisted of applying a small drop of the molybdate reagent and rubbing it over the enamel surface for 30 seconds. Using compressed air, the solution was then dried on the enamel surface to a thin moist film. A drop of the 10 per cent oxalic acid solution was applied directly over the area and rubbed in until a white precipitate formed which was then air-dried to form a white, opaque surface. To remove loosely bound precipitate, this surface was flushed with warm water while being rubbed with a cotton pledget. Using compressed air, the surface was then carefully dried again, just prior to application of a drop of an acrylic filling resin, about 5 mm. in diameter, mixed according to manufacturer's directions. After hardening, the drops were smoothed off with sandpaper disks and the patients were told to carry on their normal activities. On the control teeth, drops of water were used in place of the acids. Table I shows the results obtained.

TABLE I
ADHESION OF AN ACRYLIC FILLING MATERIAL TO TREATED AND UNTREATED LABIAL ENAMEL SURFACES INTRAORALLY

PHOSPHOMOLYBDIC–OXALIC ACID TREATMENT	UNTREATED	TREATED
Number of surfaces	15	15
Average duration of adhesion in hours	6	160

The second method of surface treatment employed 85 per cent phosphoric acid to determine the effect of a simple acid decalcification on adhesion. The method used was the same as previously, except that the drop of acid was washed off with water after the 30 seconds' contact with the surface. The results are shown in Table II.

TABLE II
ADHESION OF AN ACRYLIC FILLING MATERIAL TO TREATED AND UNTREATED LABIAL ENAMEL SURFACES INTRAORALLY

PHOSPHORIC ACID TREATMENT	UNTREATED	TREATED
Number of surfaces	10	10
Average duration of adhesion in hours	11	1,070

RESULTS

Table I compares the average number of hours that the drops of the acrylic filling material remained on the enamel surfaces of treated and untreated teeth. All the acrylics on the control teeth fell off by themselves. Only one control remained on the enamel for 24 hours; all others fell off within 12 hours. All the acrylic drops on the treated teeth adhered for more than 24 hours. Only 3 drops fell off by themselves. The others (12) had to be removed mechanically. At the request of the patients, 6 of the acrylic drops were removed within 1 week of application. If removal had not been requested, the results would have been additionally in favor of the treated surfaces. After mechanical removal of the drops, the underlying enamel appeared as a sharply defined area of clean enamel which appeared white and opaque by contrast with the surrounding enamel. In a few days, however, the treated area of the whole enamel surface appeared normal again, except for a faint loss of luster over the area of treatment.

The results of the phosphoric acid treatment (Table II) show that, whereas the controls adhered for an average of 11.2 hours before falling off, the drops on treated teeth remained on the enamel surface for an average of 1,070 hours. Of the treated teeth, 50 per cent (5) of the drops were removed mechanically. This removal required considerable force with a sharp instrument, and in about one-half of the instances it resulted in fracture of the resin rather than clean separation from the surface. The remaining 50 per cent (5) are still on the teeth at this writing, 90 days after application, in spite of repeated attempts to remove them by strong thumbnail force. After removal of the acrylic, the enamel surface appeared opaque and white as described previously, but the appearance returned to normal in a few days. No staining was evident in the treated area.

DISCUSSION

The increased adhesion obtained intraorally on treated enamel surfaces may be due to several factors, such as: (a) a tremendous increase in surface area due to the acid etching action; (b) the exposing of the organic framework of enamel which serves as a network, in and about which the acrylic can adhere; (c) the formation of a new surface due to precipitation of new sub-

stance, for instance, calcium oxalate, organic tungstate complex, and so on, to which the acrylic might adhere; (d) the removal of old, fully reacted, and inert enamel surface, exposing a fresh, reactive surface more favorable for adhesion; and (e) the presence on the enamel surface of an adsorbed layer of highly polar phosphate groups, derived from the acid used.

Regardless of the mechanisms involved, however, we do know that we can increase adhesion remarkably by acid treatments. It is felt that such treatments are clinically safe. This is supported by the absence of any lasting noticeable effects on the enamel and by the knowledge that similar concentrations of phosphoric acid are used in phosphate cements. The adhesion produced by the phosphoric acid treatments seems to be stronger and more lasting than that produced by the phosphomolybdate–oxalic acid treatment. This difference might be explained by assuming that the oxalic acid produces a precipitate which is not strongly attached to the enamel surface.

The fact that we are using acids for our surface treatments lends heavy support to the idea that the adhesion is due to a great increase in surface area and that the effect may be purely a physical phenomenon, with other acids capable of producing the same result. In addition, the use of acids may increase the wettability of the surface, allowing for more intimate contact between acrylic resin and enamel, thus favoring adhesion. Another possibility is that the use of acidic phosphate containing treatment material may, in addition to increasing surface area and wettability, allow for the adsorption of highly polar phosphate groups on the enamel surface with the result that strong polar bonding to the acrylic may also result. This type of bonding would be much more desirable than one based solely on mechanical retention inasmuch as it may be considered as a form of chemical union.

At this time we feel that because evidence of this nature has not been previously reported, the reasons for the increased adhesion are less important than the finding that the adhesive bond attained on treated, as compared to untreated, surfaces survived oral conditions for relatively long periods of time. Further experiments are indicated to test the resistance of this bond to acid and bacterial action. For the duration of the experiment, the resistance of the bond to the humidity of the mouth seems to be excellent. More work is, however, indicated to evaluate moisture resistance over periods of time comparable to the service life of a restoration. Nevertheless, even at this present stage it is believed that these findings open a new approach to the problem of obtaining adhesion of acrylic materials to teeth, thereby increasing the usefulness of these materials. For instance, we foresee that the formation of good bonds, of the sort we have demonstrated, to enamel surfaces open the possibility of successfully sealing pits and fissures for purposes of caries prevention. In addition, good bonding at the enamel cavity margins would protect against secondary or marginal decay. This would be particularly useful in those areas, such as in Class 3 and 5 restorations, where oral forces tending to break the bond are minimal.

SUMMARY

A simple method of increasing the adhesion of acrylic filling materials to enamel surfaces is presented. A phosphoric acid and a phosphomolybdate–oxalic acid treatment have been employed to alter enamel surfaces chemically. The phosphoric acid treatment seems to give better results and is simpler to use. The use of this type of treatment for sealing acrylics in pits and fissures as a method of caries prevention is suggested.

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EFFECTS OF FILLING MATERIALS ON THE PULP OF THE RAT INCISOR

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INTRODUCTION

THIS investigation was designed to study the effects of a variety of commonly used filling materials upon the growing dentin and the pulp of the incisor of the albino rat.

One of the important requirements of a dental filling material is that it does not harm the pulp. Investigators and clinicians have, therefore, stressed the need for a rapid and simple method of testing the effects of filling materials on the living pulp before these are used in human teeth. While histologic analysis of the effects of filling materials on the pulp in human teeth is essential to accuracy and objectivity, patients are reluctant to sacrifice teeth for experimental purposes. On the other hand, animal experiments permit the accumulation of relatively large numbers of specimens within a relatively short time. Most important, the conditions of testing can be more accurately controlled. The experiments can then be replicated until the variables are determined and the effects of each material on the pulp can be assessed in an objective and quantitative manner.

Until very recently, the dog was the experimental animal of choice, with occasional studies in monkey and man. In 1952, one of us (I. S.) suggested the use of the rat incisor and molar for the analysis of the effects of filling materials on the young and adult pulp. One of the objectives of this study was to establish the validity of using the rat incisor for the bio-assay of new filling materials introduced to operative dentistry.

METHODS AND MATERIALS

This study is based on the histologic investigation of the incisors of 74 male albino rats 50 to 56 days of age, weighing from 150 to 175 grams. Fourteen animals were sacrificed 2 days and 60 were sacrificed 7 or 8 days following cavity preparation and insertion of the filling.

The rats were anesthetized prior to operation by an intraperitoneal injection of 0.5 c.c. of a 1 per cent Nembutal solution. A cavity was then prepared with the aid of a binocular dissecting microscope at a magnification of ×9, on the lateral aspect of the left mandibular incisor inferior to the mental foramen in each animal and a filling was inserted (Fig. 1). A cavity approximately 2 mm. in diameter and 0.75 mm. in depth was first prepared in the bone

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at the exposed site using a No. 703 fissure bur held in a firmly fixed dental handpiece. The cavity in the dentin was then prepared using a No. 33½ inverted cone bur in the fixed handpiece (Fig. 2). The method of cavity preparation was similar to the one described by Mohammed (1952), and Schour and Mohammed (1953). The right mandibular incisor was used as the unoperated control.

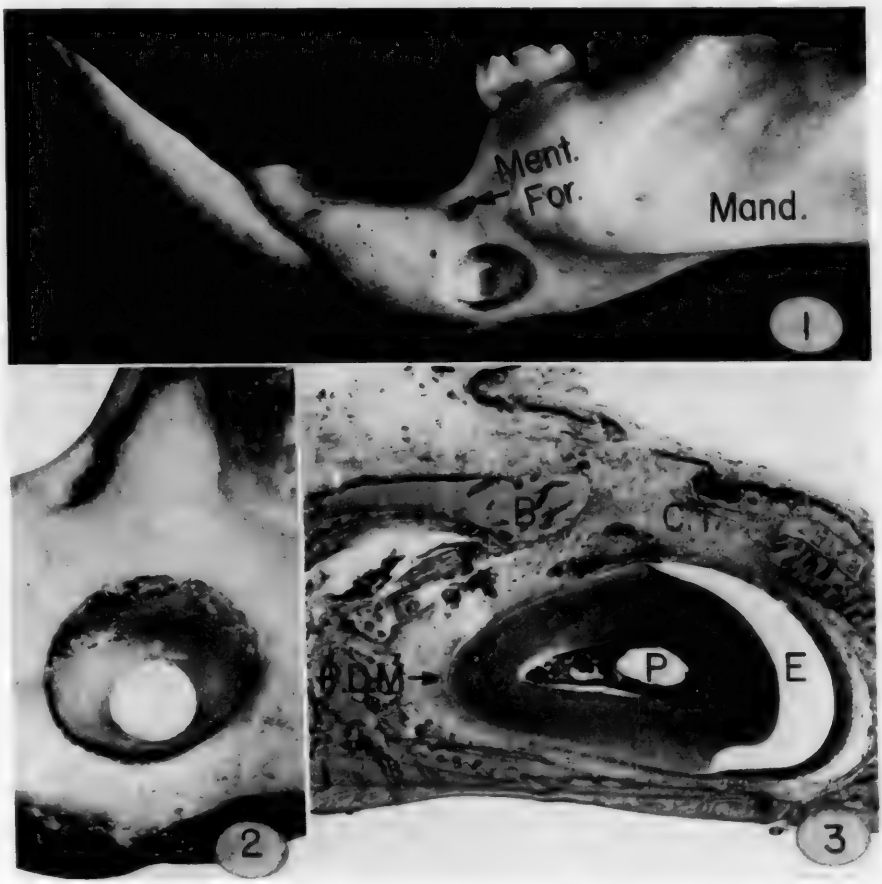

Fig. 1.—Dissected and macerated mandible showing cavity preparations in the bone and dentin of a rat's incisor. *Mand.*, Mandible; *Ment. For.*, mental foramen.

Fig. 2.—Dentin cavity filled. A layer of wax will be applied over the filling and the bone cavity will be filled with Aquadont.

Fig. 3.—Photomicrograph of a cross section of the incisor of a rat subjected to sham operation (7 days' survival). A cavity was cut into the bone (*B*) overlying the tooth but the tooth was not touched. Note the cavity in the bone overlying the tooth which is filled with connective tissue (*C.T.*) and the acute calcio-traumatic reaction (*C*) to the operation. *P*, pulp; *P.D.M.*, periodontal membrane; *E*, enamel space (×40; reduced ⅙).

Filling Procedure.—When the cavity was completed, it was washed with distilled water, dried with air, and filled with the selected filling material (Fig. 2). Each material was manipulated according to the manufacturer's specifications.

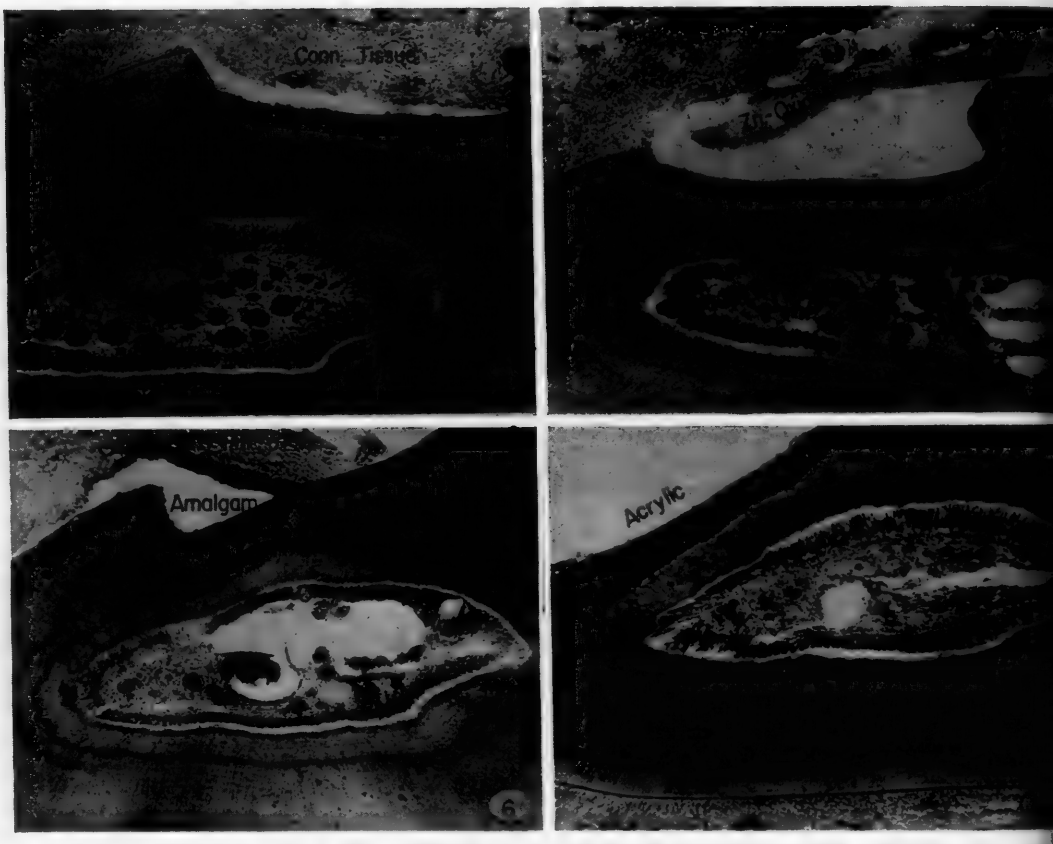

Fig. 4.—Photomicrograph of a cross section of a rat's incisor with a moderately deep cavity preparat left unfilled for 7 days. Note the ingrowth of vital connective tissue (*Conn. Tissue*) into the cavity. T calcio-traumatic band (*C*) is only slightly prominent (1). The stratification of the postoperative dentin (D_1) within normal limits. Odontoblasts (*O*) normal (×120; reduced ⅓).

Fig. 5.—Photomicrograph of a cross section of a rat's incisor with a deep cavity preparation filled w zinc oxide-eugenol cement (*Zn. Oxide-Eugenol*) and sacrificed 7 days later. Note the prominent (2) calc traumatic band (*C*) under the cavity and its lesser prominence (1) in the rest of the dentin. T postoperative dentin (D_2) shows a normal degree of calcification and prominence of the incremental patt (×120; reduced ⅓).

Fig. 6.—Photomicrograph of a cross section through a rat's incisor with a shallow cavity filled with sil amalgam (7 days' survival). Note staining of the dentin under the floor of cavity by the amalgam. The c cio-traumatic band (*C*) formed at the time of operation is mildly accentuated (1) both locally and genera The postoperative dentin (D_2) is normal in quality and quantity. Note the disturbance in the odontoblastic la (*O*) under the cavity. The engorged vessels in the pulp are characteristic, but may be an artifact caused shrinkage (×120; reduced ⅓).

Fig. 7.—Photomicrograph of a cross section through a rat's incisor with a moderately deep cavity fil with a self-curing acrylic (Kadon) (8 days' survival). Note the strongly accentuated (2) calcio-traumatic ba (*C*) under the cavity preparation and the severe disturbance in the postoperative dentin (D_2), which is poo calcified, almost completely atubular and amorphous in structure (6). Note the calcio-traumatic band a disturbed calcification in the postoperative dentin opposite the cavity preparation. In spite of the sev changes in the structure and calcification of the postoperative dentin under the cavity, the disturbance in t morphology and arrangement of the odontoblasts (*O*) is relatively slight (1) (×120; reduced ⅓).

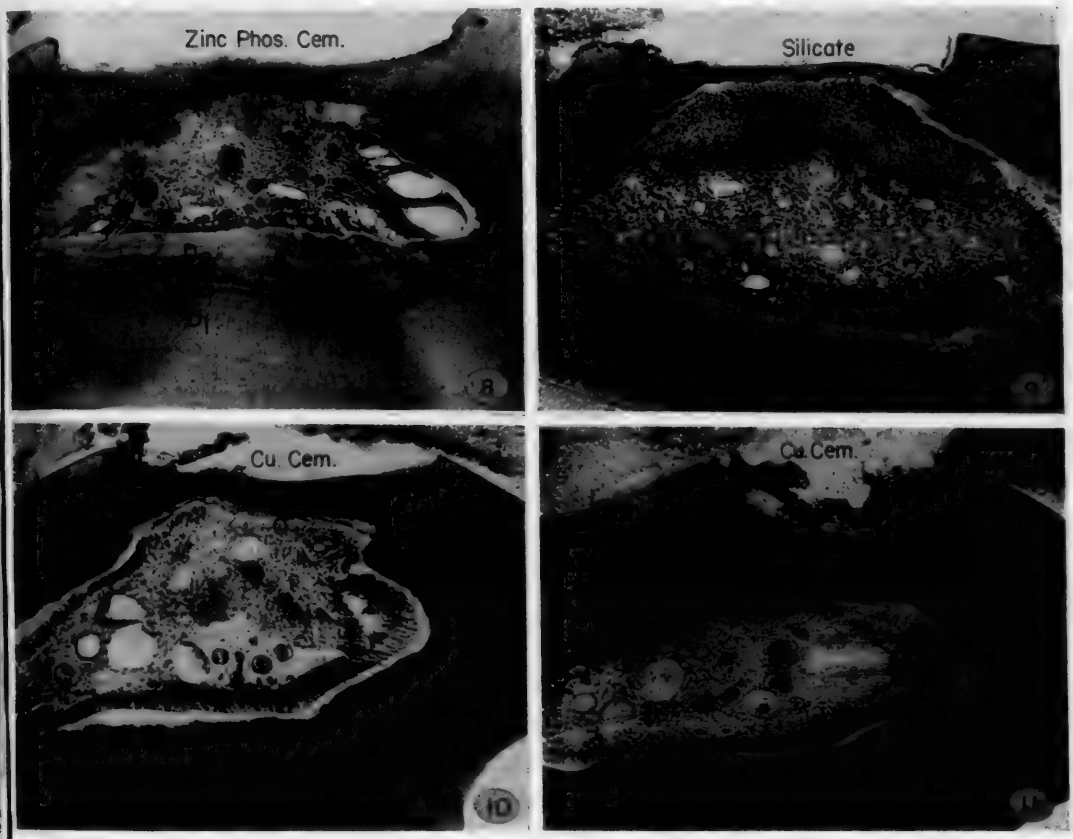

Fig. 8.—Photomicrograph of a cross section through a rat's incisor with a medium cavity filled with zinc oxyphosphate cement (7 days' survival). Note the severe damage (3) to the odontoblasts under the cavity, and the dentin hypoplasia (3–). The dentin which is formed is atubular and amorphous (6). The effects of the filling material are not confined to the cavity area alone. Note the strongly accentuated (2) calcio-traumatic reaction and disturbed calcification (4) of the postoperative dentin (D_2) formed on the side opposite the cavity. The amount of dentin formed on the side opposite the cavity is slightly increased (N+) (×120; reduced ⅓).

Fig. 9.—Photomicrograph of a cross section through a rat's incisor with a deep cavity filled with silicate (8 days' survival). Note the severe destruction (3) of the odontoblasts under the cavity preparation, the dentin aplasia (4–), and the walled-off abscess (*A*) in the pulp. Note also the severe disturbance in dentin formation and structure (6) on the side opposite the cavity (×120; reduced ½).

Fig. 10.—Photomicrograph of a cross section through a rat's incisor with a moderately deep cavity filled with copper cement (7 days' survival). Note the complete destruction of odontoblasts under the cavity and complete cessation of postoperative dentin formation (aplasia) (×120; reduced ½).

Fig. 11.—Photomicrograph of a cross section through a rat's incisor with a medium cavity which was lined with a calcium hydroxide base (Chembar) prior to insertion of copper cement (7 days' survival). Compare with Fig. 10. Note the lesser damage to the odontoblasts (*O*) and the normal amount of postoperative dentin formation (D_2) under the cavity (×120; reduced ½).

After the filling was set, it was covered with a layer of wax approximately equal to the thickness of the periodontal membrane. The cavity in the bone was then filled in with a thick mixture of Aquadont. The interposition of a layer of wax between the dentin filling and the bone filling was necessary in order to prevent ankylosis of the tooth to the bone and permit the eruption of the incisor. It further served to seal the dentin filling against displacement and ingress of tissue fluids.

The entire operation occupied approximately 40 minutes.

Filling Materials Used.—The filling materials used were: (1) zinc oxide and eugenol; (2) silver amalgam and mercury; (3) self-curing acrylic (Kadon); (4) silicate; (5) zinc oxyphosphate cement; (6) black copper oxyphosphate cement; (7) cavity varnish; (8) calcium hydroxide liner (Chembar); and (9) Aquadont for filling the cavity in the bone. Aquadont consists of zinc oxide two parts, zinc sulfate one part, and corn starch one part, and sets rapidly when mixed with water.

Each filling material was tested in groups of 5 to 14 animals each (Table I). In 9 animals the cavities were left unfilled and in 2 animals a sham operation was performed, cutting through the bone only and leaving the dentin untouched (Fig. 3).

Duration Of Experiments.—The animals were sacrificed after 2 and 7 or 8 days by ether and decapitation. The maximal survival period was limited to 8 days since the rat incisor is a continuously growing and continuously erupting tooth. After 8 days, the operated portion of the dentin and the subjacent area of the pulp reach the anterior portion of the tooth where the pulp begins to show aging changes and to degenerate so that the experimental effects become obscured.

Histologic Preparation.—After the animals were sacrificed, the mandibles containing the lower incisors were dissected and fixed in 10 per cent neutral Formalin, decalcified in 5 per cent nitric acid for 72 hours, and histologic sections cut on a freezing microtome at 25 microns, using carbon dioxide gas. All sections were stained routinely with hematoxylin and eosin.

METHODS OF ANALYSIS

The effects of the filling materials upon the dental pulp were assessed by analyzing the character and amount of dentin produced by the odontoblasts after the filling was inserted. The character and amount of dentin are a very delicate indicator of the physiology of the odontoblasts, often revealing alterations in cell function long before cytologic changes can be discerned.

The Calcio-traumatic Reaction.—The immediate effect of the operative procedure and filling was the formation of a distinct calcio-traumatic band which reflects a disturbance in the calcification of the predentin formed at the time of operation (*C*, Figs. 3 to 11). It consists of a very thin, barely discernible hypercalcified (deep blue) line followed by a wider hypocalcified stripe (light pink) and a hypercalcified (light blue) stripe.

The calcio-traumatic band could easily be located by counting the number of daily incremental lines formed postoperatively. However, in almost every

instance the acute calcio-traumatic effect was sharply prominent and stood out clearly between the pre-experimental and postoperative areas of dentin formation (Figs. 4 to 11).

Postoperative dentin calcification and formation varied from complete recovery (normal postoperative dentin formation and calcification), to various degrees of disturbance in calcification (accentuated stratification; interglobular dentin), to changes in the structure of the dentin (loss of tubular structure), to interferences in the amount of dentin deposited (dentin hypoplasia or aplasia).

The amount of postoperative dentin was measured from the acute calcio-traumatic band formed at the time of operation to the pulpal surface. The amount deposited under the area of the cavity preparation was compared with the amount formed on the side opposite the cavity preparation and in the unoperated right incisor.

The average daily rate of dentin deposition at the level of the cavity preparation in this group of experimental animals was 12.12 microns with a standard deviation of 1.72 microns. These measurements indicated that the "normal" amount of dentin formed after 7 days would be from 73 to 97 microns (85 ± 12). An increase or decrease was considered to be significant only when it was greater than 2.5 standard deviations (± 30 microns) (labeled 2+ or 2– in the tables). A difference between the amount of dentin formed under the cavity preparation and the amount on the opposite side which was less than 1.5 standard deviations (18 microns) was considered not significant. Differences between these (19 to 29 microns) were considered as of borderline significance and labeled as N+ or N–.

Controls.—The dentin immediately under the cavity preparation was compared with the unoperated dentin in the same tooth on the surface immediately opposite to the operated dentin. In addition, the unoperated lower incisor on the right side was sectioned at the equivalent level near the mental foramen and studied. This unoperated tooth served as a negative control.

CRITERIA USED FOR ANALYSIS

The following criteria were used to analyze the effects of the filling materials on the function of the odontoblasts:

Acute Calcio-traumatic Band.—

0—None present, indicating no reaction to operative procedure.

1—Mild reaction. Slight accentuation of the incremental line formed at the time of operation. Hypercalcified stripe prominent, hypocalcified stripe faint.

2—Moderate reaction. Prominent calcio-traumatic band. The hypocalcified stripe wider and more prominent than the hypercalcified stripe.

Changes in the Postoperative Dentin.—

(A) *Calcification:*

N—Calcification and structure of dentin normal.

1—Slight disturbance in calcification characterized by a slight accentuation in the incremental pattern. Within "normal" limits.

2—Definite disturbance in calcification. The incremental pattern very prominent, beyond normal limits.

3—Moderate disturbance in calcification. The calcification of the matrix behind the incremental lines not homogeneous but granular.

4—Severe disturbance in calcification. Interglobular calcification of dentin with or without accentuation of incremental lines.

(B) *Structure:*

5—Decreased number of tubules in dentin.

6—Atubular dentin. Stains a pale blue with hematoxylin-eosin. Occasional cellular inclusions.

Amount of Postoperative Dentin Formation.—

N—Normal.

N+—Increased slightly. Not statistically significant.

N–—Decreased slightly. Not statistically significant.

2+—Definitely increased. Statistically significant (more than 2.5 S.D.).

2–—Definitely decreased in amount. Statistically significant.

3–—Dentin hypoplasia. Localized death of cells with interrupted and irregular formation of dentin.

4–—Dentin aplasia. Complete or almost complete inhibition of dentin deposition under area of cavity preparation.

Changes in Odontoblastic Morphology.—

N—Normal in morphology, number, and arrangement in pulp.

1—Odontoblastic layer in pulp slightly disarranged.

2—Severe disarrangement of odontoblastic layer in pulp.

3—Degeneration and loss of odontoblasts.

FINDINGS

The findings of this study are summarized in Table I.

Effects Of Operation per se.—Two animals were subjected to sham operation to determine the effects of the operation per se. Cavities were prepared in the bone only, without injury to the dentin.

A calcio-traumatic band was found to be present at the level of the dentin, forming and calcifying at the time of the operation (Fig. 3). It was only slightly accentuated. The incremental lines in the postoperative dentin were slightly more prominent than the lines in the preoperative dentin. This could not be considered as abnormal since a mild accentuation of the incremental lines in the dentin is often found in unoperated, normal animals. It reflects the extremely delicate response of the odontoblasts and the calcifying dentin to relatively minor systemic episodes in the course of the animal's metabolism. The dentin was otherwise normal in amount and in structure.

The calcio-traumatic reaction was probably the result of the anesthesia and the failure to eat following the operation.

Effects Of Cavity Preparation per se.—In order to determine the effects of the cavity preparation per se upon the function of the odontoblasts, cavities were prepared in the incisors of 14 animals and left unfilled. Because of the

anatomic peculiarity of the incisor of the rat, the cavity preparations were made through the alveolar bone and periodontal membrane. The unfilled cavities were not exposed to the action of the saliva as is the case in man and in dogs but became filled by a blood clot into which connective tissue proliferated from the periodontal membrane (Fig. 4). Two cavities were covered with gold shields to prevent this ingrowth of connective tissue in order to analyze the effects of the connective tissue filling on the function of the odontoblasts.

TABLE I
EFFECTS OF OPERATIVE PROCEDURES AND FILLING MATERIALS ON THE PULP OF THE INCISOR OF THE ALBINO RAT*

NO. OF ANIMALS	FILLING MATERIAL	CAVITY DEPTH	CALCIO-TRAUM. BAND	POSTOPERATIVE DENTIN		ODONTOBL.	REMARKS
				CALCIF. AND STRUCTURE	AMOUNT FORMED		
2	Bone cavity only (sham operation)	None	1	1	N	N	
14	Open cavity filled with conn. tissue	Shallow	1	N	N	N	Minimal effect
		Medium	1	N	N	N	
		Deep	1	N	N+	N	
2	Open cavity covered with gold plate	Shallow					Effect more pronounced in the absence of connective tissue
		Medium	2	1	N	N	
		Deep	2	1	N+	N	
8	Zinc oxide and eugenol	Shallow	0	N	N	N	Palliative effect with rapid recovery of odontoblasts
		Medium	1	N	N	N	
		Deep	2	N	N	N	
8	Amalgam	Shallow	1	N	N	N	Penetration of metallic ions with localized damage to odontoblast
		Medium	2	1	N	1	
		Deep	2	5–	2–	2	
10	Self-curing acrylic (Kadon)	Shallow	2	3	N+	N	Acute effects severe and cumulative with time
		Medium	2	4	N+	1	
		Deep	2	5	2+	1	
5	Zinc oxyphos. cement	Shallow	1	N	2+	1	Dentin hypoplasia in deep and occasionally medium cavities
		Medium	2	6	2–	3	
		Deep	Hypoplasia		3–	3	
14	Silicate cement	Shallow	2	4	3–	3	Marked injury and death of odontoblasts. Abscess in pulp
		Medium	Hypoplasia		3–	3	
		Deep	Aplasia		4–	3	
11	Black copper cement	Shallow	Aplasia		4–	3	Odontoblasts entirely destroyed (aplasia)
		Medium	Aplasia		4–	3	
		Deep	Aplasia		4–	3	
74							

*See text for abbreviations used in this table.

Effects in cavities filled with vital connective tissue: A faint calcio-traumatic band was formed at the time of operation. The postoperative dentin showed normal calcification (Fig. 4). Neither effect was significantly different from those observed following the sham operation when the dentin was left untouched (Table I).

Effects in cavities covered with gold shields: These specimens showed a more prominent accentuation of the calcio-traumatic band formed at the time of operation than in the specimens filled with vital connective tissue. The calcification of the postoperative dentin was also somewhat more prominently

stratified than in the previous specimens. This indicated that the blood clot which filled the cavity preparation, and the connective tissue which grew in subsequently, probably exerted a palliative effect on the odontoblasts and pulp.

There was a tendency in deep cavity preparations for more dentin to be deposited under the cavity area than on the opposite side, or in the unoperated control tooth in both series described in Table I. However, while this increase was consistent, it was too small (5 to 10 microns) to be statistically significant. Nonetheless, the impression remained that the irritant effects of the deep cavity preparation per se produced a slight stimulating effect upon the odontoblasts to produce a slightly greater amount of dentin under the cavity.

Effect of Depth of Cavity Preparation.—The depth of the cavity preparation and the distance of the filling material from the pulp greatly influenced the reaction of the odontoblasts. It was observed that the deeper the cavity preparation, the more severe the damage to the activity of the odontoblasts and the poorer the quality of the dentin formed by these cells. Cavity preparations which extended into the pulpal third of the dentin width usually resulted in some damage to the odontoblasts since, in almost every instance, the calcification of the dentin formed postoperatively showed some deficiency. Cavity preparations which extended only into the middle third of the dentin width were much less injurious than those closer to the pulp, while cavities limited to the peripheral third of the dentin showed the least damage to the pulpal cells and interference with dentin formation and calcification.

For this reason, all specimens were grouped and analyzed according to the distance of the cavity base from the *original* pulpal surface. The location of the original pulpal surface was determined to be at the level of the calcio-traumatic line invariably found at the junction of the pre- and postexperimental dentin. All measurements were thus made from the floor of the cavity to the calcio-traumatic line in a perpendicular direction.

Cavity preparations which reached into, but not beyond, the middle third of the dentin (distance from floor of cavity to the original pulpal surface from 55 to 85 microns) were considered to be moderate in depth. This was the standard depth at which the effects of different filling materials were compared. If the floor of the cavity reached into the pulpal third of the dentin, it was considered to be deep (15 to 50 microns from the original pulpal surface). Preparations in the peripheral third of the dentin were referred to as shallow (more than 90 microns).

Effects of Zinc Oxide-Eugenol Cement.—In moderately deep cavities, the reaction to zinc oxide-eugenol cement was slightly more pronounced than in the specimens filled with connective tissue, but less pronounced than in the specimens in which the cavities were covered with a gold plate (Table I). The calcio-traumatic band formed at the time of operation appeared midway in prominence between the other two groups. The postoperative dentin was generally normal, but occasional specimens showed a distinct prominence of the incremental

pattern. The stratification was slightly more prominent than that seen under the cavities of equivalent depth filled with connective tissue but significantly less than under the cavities covered with a gold plate (Table I).

In shallow cavities, the acute calcio-traumatic and postoperative reactions were indistinguishable from those observed in specimens with cavities filled with connective tissue. In two specimens these reactions were entirely absent, suggesting a palliative influence by the eugenol on the injured odontoblasts. In the deeper cavities, the acute calcio-traumatic reaction was definitely more pronounced. However, the calcification of the postoperative dentin and the odontoblasts appeared to be normal even in very deep cavities (30 microns from the pulp) (Fig. 5).

These findings indicate that zinc oxide-eugenol had a definite palliative effect upon the odontoblasts and tended to nullify the effects of the cavity preparation.

Effects of Amalgam.—Silver amalgam fillings produced a characteristic staining of the underlying dentin, as if metallic ions had diffused from the amalgam into the dentinal tubules in a manner similar to that described in human teeth (Fig. 6). This effect could be observed even in specimens surviving only 2 days. This indicates a much greater permeability of the dentin in the rat incisor than in human teeth since, in the latter, the permeation of metallic ions from the amalgam requires months of time.

The reaction of the odontoblasts to the amalgam was more pronounced than in any of the previous specimens (Table I and Fig. 6). The acute calcio-traumatic reaction was prominent even in shallow cavities. In medium cavities, the calcio-traumatic reaction was very prominent while the 7 incremental lines formed postoperatively were definitely accentuated (Fig. 6). The effect of the amalgam on the odontoblasts was obviously more severe than that observed in empty cavities covered with a gold plate, and substantially more severe than the effect of the zinc oxide-eugenol cement, even when the latter was placed in very deep cavities (Table I).

The odontoblasts under medium cavities were disarranged and atrophied, indicating severe injury to these cells. Most of the specimens showed severe changes in the pulp which were suggestive of hemorrhage. However, these changes may be artifacts caused by shrinkage and must await future clarification.

In specimens with deep cavity preparations, the metallic ions, as evidenced by the stain, penetrated to the pulp and produced severe disarrangement of the odontoblastic layer. The postoperative dentin formation was markedly deficient in amount (2–) and in quality (poorly calcified and deficient in tubules). The depth of penetration of the metallic stain was directly related to the site and degree of injury to the odontoblasts.

It was quite evident that amalgam placed in deep cavities produced severe injury to the odontoblasts. This injury was apparently not due to thermal shock since in these specimens all fillings were covered by a layer of wax plus a thick layer of Aquadont in the bone cavity. Severe injury resulted only in those areas where the metallic stain reached the cell bodies of the odontoblasts.

Effects of Self-curing Acrylics.—The reaction of the odontoblasts to the self-curing acrylic (Kadon) applied by the brush method was more severe than to amalgam (Table I). Shallow cavity preparations filled with a self-curing acrylic resulted in the appearance of a very prominent calcio-traumatic band formed at the time of operation followed by a definite accentuation of the incremental pattern in the postoperative dentin (Table I). In specimens with moderately deep cavities, the postoperative dentin was interglobular, indicating a severe deficiency in calcification. The odontoblasts were disarranged. Under the deeper cavity preparations, the dentin was atubular and sometimes entirely amorphous (Fig. 7).

In shallow cavities, the effects of the acrylic material were confined to the area subtended by the preparation. In moderately deep cavities, the effects extended peripherally to adjacent odontoblasts outside the cavity area, while in very deep cavities, even the cells opposite the cavity preparation were affected (Fig. 7). This indicated that the effects of this material were not limited to the injured cells under the cavity preparation but that it (the monomer?) could penetrate into the substance of the pulp to affect distant odontoblasts.

The injurious effects produced by the self-curing acrylic fillings were found to be cumulative since the changes described previously increased in severity with time. The changes in the postoperative dentin were much more severe in specimens surviving 7 days than in those sacrificed after 2 days.

The amount of dentin formed under acrylic fillings was increased. Measurements indicated that this increase was consistent and approached statistical significance—but did not quite reach this level, except occasionally under deep cavities. The impression was that the irritant effects of the acrylic resulted in an increased amount of dentin formation. However, this dentin was of poor quality, always poorly calcified, frequently atubular, and sometimes even amorphous in character (Table I and Fig. 7).

Effects of Zinc Oxyphosphate Cement.—The effects of zinc oxyphosphate cement upon odontoblastic function were more severe than in any of the previous specimens when placed in medium or deep cavities (Table I). In shallow cavities (more than 100 microns from the pulp), the alignment of the odontoblasts under the cavity was disturbed. The postoperative dentin was normal in quality but significantly increased (2+) in amount (Table I). This indicated a stimulating effect on odontoblastic function in the shallow cavities.

In moderately deep cavities, however, the effects were very injurious (Fig. 8). The calcio-traumatic band formed at the time of operation was prominent and the postoperative dentin was heavily stratified and irregular, atubular and amorphous in structure. The odontoblasts were severely disarranged and reduced in number. Dentin formation was significantly deficient (2–) in amount (Table I). Interestingly, dentin formation on the side opposite the cavity was often stimulated (Fig. 8). Engorged vessels and inflammatory cells were often observed in the pulp.

In deep cavities, zinc oxyphosphate cement produced death of many odontoblasts and dentin hypoplasia (Fig. 8).

Effects of Silicate.—The effects of silicate on the pulp under the conditions of this experiment were very severe (Table I). The odontoblasts were severely injured or destroyed (Coy and associates), even under shallow cavities. Dentin hypoplasia was the rule in medium cavities, and in deeper cavities complete aplasia (Fig. 9). When some dentin matrix was formed, it was atubular, irregular, and amorphous in structure.

In deeper cavities, the pulpal tissue under the cavity frequently showed a marked inflammatory reaction, cellular infiltration, and abscess formation. The abscesses were walled off (Fig. 9) and calcification of degenerated areas could be seen.

In shallow cavities the cells and dentin opposite the cavity preparation were only slightly affected, but in deep cavities the odontoblasts opposite the cavity were also severely injured so that dentin formation was deficient in quantity (hypoplastic) and in quality (interglobular and atubular). The effects seen in shallow cavities were considerably more injurious than those observed under amalgam and acrylic fillings in even the deepest cavities and more injurious than the effects of zinc oxyphosphate cement placed in moderately deep cavities (Table I). The widespread effects of the silicate fillings upon the cells of the pulp outside the cavity area were very striking and much more extensive than those observed under zinc oxyphosphate cement fillings. The effects observed under acrylic fillings were much milder and less extensive by comparison, while the effects under amalgam fillings were limited entirely to the cut tubules even in very deep cavity preparations.

Effects of varnish under silicates: In six specimens, varnish was applied to the cavity prior to filling with silicate. No significant differences could be observed in these specimens when compared with those in which varnish was not used. These results confirm similar findings by Zander (1946) and Zander, Glenn, and Nelson (1947), and do not support the common clinical practice of using a varnish to protect the pulp prior to filling with silicate.

Effects of Black Copper Cement.—The most severe changes in this series were effected by the use of black copper cement. Even in a very shallow cavity preparation, the subtended odontoblasts were almost completely destroyed so that practically no dentin was formed under the cavity thereafter (aplasia). In moderately deep cavities (65 to 75 microns from the pulp) the odontoblasts were completely destroyed, and complete aplasia resulted. The pulpal tissue underlying this area was also disarranged (Fig. 10).

The severe effect of the black copper cement was apparently very acute and very severe but tended to be localized to the area under, and immediately adjacent to, the cavity outline. The dentin formed outside the cavity outline was fairly normal and not severely stratified, or altered in amount as occurred in teeth filled with silicate (compare Figs. 9 and 10).

Effects of a Calcium Hydroxide Base.—A base material consisting of calcium hydroxide dispersed in a solution of polystyrene in chloroform (Chem-

bar) was used in four cavity preparations under a filling of black copper cement to test its efficacy in protecting the pulp against the noxious influences of the cement.

It was found that when Chembar was used in shallow cavities filled with black copper cement, the expected injurious effects by the copper cement were greatly reduced (Fig. 11). A calcio-traumatic band was formed at the time of operation but the postoperative dentin was almost normal in quality and quantity.

In moderately deep cavities, Chembar was less completely effective. The severe effects previously described under fillings with copper cement were much reduced, but the odontoblasts were disarranged and the postoperative dentin was deficient in amount and defective in tubular structure (Fig. 11). However, in no instance was the effect as destructive as that described for comparable cavities filled with the black copper cement alone. The protective action of Chembar, when placed under silicates, has been demonstrated by Zander and Pejko (1947).

DISCUSSION

Use of the rat for the bio-assay of filling materials offers many advantages over the use of the dog and monkey. First, the cost and time involved are much less. More important, the rate of dentin formation is much more rapid than in the dog or monkey. Because of this, the dentin-forming cells are very active and, therefore, also much more responsive to minor irritants and insults. These, in turn, are clearly and indelibly recorded as delicate variations in calcification and structure of the rapidly forming dentin (Schour and Massler, 1942). The kymographic quality of the dentin in the incisor of the rat is superior to that of the teeth of the dog or monkey. Further, although the response of the pulp of the rat incisor is delicate and rapid, it is relatively constant and reproducible. This is important since, although dogs have been the animals used by the majority of investigators, the reaction of the pulp to filling materials placed under standardized conditions tends to show rather wide variations—presumably because of the heterogenous nature of the animals in respect to age, nutrition, and genetic background (Zander, 1951).

The results obtained in this single study reproduce the combined findings obtained in human teeth by many investigators (Fish, 1931; Manley, 1944; Shroff, 1947; Zander, 1946, 1947; Fisher, 1949; Castagnola, 1950; Kramer and McLean, 1952), and in other animals by Fascioli, 1924; Dixon, 1934; Fish, 1932; Gurley and Van Huysen, 1940, 1942; James and Diefenbach, 1942; Coy, Bear, and Kreshover, 1952; and many others (see References). This study thus demonstrates the validity of using the incisor of the albino rat to test the biologic response of the pulp to new (and old) filling materials.

For the study of chronic, long-term effects, cavities can be prepared in the molars of the same animal as demonstrated by Maurice (1953).

The only objections raised to the use of the rat for this purpose is the small size of the tooth, which requires a meticulous technique of cavity prepara-

tion under a stereomicroscope. The development of a finely calibrated instrument to produce cavities of standard size and depth would greatly aid this research.

Clinical Applications.—The translation of results obtained in experimental animals to the clinical situation in man should always be made with caution. This is true even when these results have been confirmed by histologic investigations in the teeth of man. Human pulps react in a variable manner even under apparently similar clinical conditions. Whether this is due to age factors or to inevitable biologic variations is not clear. Furthermore, human teeth are usually carious before the cavity is prepared and the filling is placed. The reaction of the dentin and the pulp is, therefore, greatly modified by the effects of the caries (sclerotic dentin and secondary dentin formation) as well as the clinical management of the lesion (sedative dressing prior to filling, speed and depth of cavity preparation, sterilization of the dentin, and so forth). However, it should be pointed out that in all cavity preparations, extension for prevention inevitably opens tubules not previously attacked by caries. In such areas the present findings may well apply.

This study indicates that the dentin in the rat incisor is thinner and more permeable than the dentin in human teeth and that the pulp is therefore more reactive to fillings placed in these teeth. Nonetheless, the relative effects obtained in the rat incisor are very similar to those obtained under experimental conditions in young, noncarious human teeth by other investigators. This indicates that the results obtained in the rat incisor may be translated to the situation in the young pulps of children, if this translation is made with caution and gives full consideration to the modifications created in the dentin and the pulp by caries and the preceding clinical management of the lesion. It would be desirable to investigate the effects of these filling materials on the pulp when placed in dentin previously attacked by caries and over pulps in which secondary dentin has already been formed.

SUMMARY AND CONCLUSIONS

Cavities were prepared in the incisors of 74 albino rats and filled with a variety of filling materials to determine the effects of these materials on the young pulp and odontoblasts. The effects on the odontoblasts were evaluated primarily from alterations in the quality (calcification and structure) and quantity of the dentin formed after the operation. Animals were sacrificed 2, 7, and 8 days after operation.

The operative procedure per se produced an acute calcio-traumatic reaction from which the odontoblasts almost completely recovered as evidenced by the mild accentuation of the normal incremental pattern during the 7 days of postoperative dentin formation when the cavities were left unfilled.

Filling the cavity with zinc oxide and eugenol or allowing vital connective tissue to grow into the cavity resulted in a reduction in the acute operative injury to the odontoblasts and accelerated the postoperative recovery as evi-

denced by the formation of a less prominent calcio-traumatic band and a return of the postoperative incremental pattern to normal (Figs. 4 and 5). No morphologic changes were observed in the pulp or the odontoblasts.

Insertion of a silver amalgam filling produced a definite localized injury and atrophy of the subtended odontoblasts. The injury was markedly aggravated as the depth of the cavity increased and the filling (and its diffusing metallic ions) came closer to the pulp and the cell bodies of the odontoblasts (Fig. 6).

Self-curing acrylic was slightly more injurious to the odontoblasts than the amalgam, as evidenced by the more severe acute calcio-traumatic reaction and the postoperative formation of a slightly greater amount of dentin of poor quality (interglobular under shallow cavities and atubular under deep cavities) (Fig. 7). The injury to the cells was cumulative and progressive as well as more extensive than the strictly localized effects by the amalgam.

Filling with zinc oxyphosphate cement, silicates, and copper cements produced increasingly severe injury and death of the odontoblasts with resulting defects in the quantity as well as quality of the dentin (Figs. 8 to 10). These effects were increasingly severe in the order of materials listed (Table I) and as the depth of the cavity increased. Zinc oxyphosphate cement in medium and deep cavities resulted in death of many, but not all, odontoblasts, and in an inhibition of dentin formation (Fig. 8). Silicates produced, in addition to the death of many odontoblasts and dentin hypoplasia, inflammation and abscess formation in the pulp itself (Fig. 9). Copper cement was most destructive, resulting in complete dentin aplasia even in shallow cavities (Fig. 10). The effects of the silicates extended beyond the limits of the cavity. The effects of the oxyphosphate cements were more localized.

Cavity varnish was found to be completely ineffective in protecting the pulp against injury by silicates. A calcium hydroxide liner was found to be much more effective in this respect (Fig. 11).

The results obtained in this study are compatible with similar studies made in human teeth, and in teeth of other animals by numerous investigators. The results obtained in the present study are more definite and striking because the rat incisor is a rapidly growing tooth and, therefore, very reactive to the local injuries produced by the cavity preparation and fillings. The reactions of the very active odontoblasts to the filling materials were clearly imprinted in the dentin forming and calcifying after the fillings were placed and these effects could be studied in greater detail under much more rigidly controlled conditions than can be obtained in man. The results obtained indicate that the rat incisor has many advantages over the teeth of dogs or monkeys for the assessment and evaluation of new filling materials used in dentistry.

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DENTAL CARIES IN CHILDREN ONE TO SIX YEARS OF AGE AS RELATED TO SOCIOECONOMIC LEVEL, FOOD HABITS, AND TOOTHBRUSHING

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INTRODUCTION

DURING 1951, we examined 650 children, 1 to 6 years of age, living in Portland, Oregon, for dental caries experience. Children of this young age level could be reached only at different nurseries, kindergartens, and grade schools. The staffs at different nurseries revealed that some of the nurseries were partly financed by charitable organizations, and others were maintained privately by the fees paid by the parents. We surmised that this factor would select the children from two different socioeconomic levels. Studying the dental caries incidence of children, according to the nursery attended, revealed a significant difference.[4] However, the method used for determining the socioeconomic status of parents from the characteristics of the nursery (whether receiving public or private support) was criticized by sociologists on the basis of being poor in distinguishing socioeconomic levels. It was recommended that greater accuracy in establishing socioeconomic status of parents would be obtained if a trained interviewer visited the home of each child and, using a standardized questionnaire, secured information from the parents.

The present investigation was therefore planned to study the association of dental caries in children with the socioeconomic status of parents. The possibility of the differences in socioeconomic status of parents resulting in variation in the frequency of refined carbohydrate consumption and health education of children (brushing of teeth) was also considered and investigated in this study.

REVIEW OF THE LITERATURE

Mellanby and Mellanby[1] made a study of the dental caries incidence of the 5-year-old children in 33 public schools in London. The number of caries-free children decreased from 6.1 per cent in the schools of higher income districts to 2.8 per cent in the schools of the poor districts, while the average caries figure increased from 4.4 per cent to 4.9 per cent.

Cohen and Green[2] conducted a survey on approximately 500 preschool children (aged 4 and 5 years). Those in the high socioeconomic level showed the lowest def counts.

Potgieter and Everitt[3] reported a one-week study of the eating habits of 385 children, 4 to 8 years old, in 2 elementary schools. The children's diets were evaluated by comparison with the Basic Seven food guide. This report showed all but one of the diets to be medium or poor in nutritional adequacy. Children

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with "better" diets were slightly superior in physical status, in dental status, in days absent because of illness, and in educational ratings, and definitely better in social adjustment.

Dykes[4] reported on a survey of 23,000 Oakland children attending elementary, junior high, and high schools: 18.8 per cent had candy before 9 A.M.; 35.1 per cent had candy before 1 P.M.; 6.1 per cent had soft drinks before 9 A.M.; 15.3 per cent had soft drinks before 1 P.M.

MATERIALS AND METHODS

Dental examinations for 650 Portland children had already been conducted.[4] The present investigators therefore considered it expedient to secure information

TABLE I

INTERVIEW SCHEDULE USED FOR HOME INTERVIEWS TO SECURE AND RECORD INFORMATION FROM THE PARENTS OF CHILDREN EXAMINED FOR DENTAL CARIES STUDY

DENTAL SURVEY

Serial No. _____

Child's Name _____ Nursery _____

Address _____

	Yes	No
1. Does the child have desserts daily for Lunch		
Dinner		

2. Which of the following does he eat for dessert, and how many times a week?
   Cake _____ Cookies _____
   Pie _____ Candies _____
   Ice Cream _____ Fresh Fruit _____
   Jello _____ Canned Fruit _____
3. Does the child eat in between meals? Yes ____ No ____. If yes, how many times daily? _____
4. If the child brushes his teeth, how often does he do it? _____ And when?
   At rising _____ After lunch _____
   Before breakfast _____ After dinner _____
   After breakfast _____ Before retiring _____
5. Where has the family lived during the last six years?

Length of Residence	Town	State

6. How many rooms are occupied exclusively by your family in the house? No. _____
7. (a) How many members of your immediate (mother, father, children) family live with you? No. of Adults _____ No. of Children _____.
8. Does the family have a private bathroom? Yes ____ No ____. If more than one, how many? _____
9. Do you take any magazines regularly? Number _____. If so, name three. _____
10. Do you employ any domestic help? None ____ Part time _____ Full time _____.
11. What is the occupation of the father (if employed)? Please give as specific details as possible. _____
12. If the mother is working, what type of work is she doing? _____
13. How many years of school completed beyond grammar school? Father _____ Mother _____
14. Into which of the following income groups does this family fall?
    $ 0-2,000 _____ $6,001-8,000 _____
    2,001-4,000 _____ 8,001-over _____
    4,001-6,000 _____
15. Recorder's estimate _____ Recorder _____

regarding the socioeconomic status of the parents as well as the food and oral hygiene habits of the same children.

Table I illustrates the questionnaire used by the interviewers. The interviewers were 3 graduate students who have had considerable experience in interviewing of this kind. Questions 1, 2, and 3 secured information about the types of foods and frequency of eating. Question 4 obtained information about the toothbrushing habits. Question 5 was used to find out if the child had lived in a fluoride endemic area. Questions 6 to 14 were used to determine the socioeconomic status of the parents. The information obtained from Questions 6 through 14 overlapped and aided in double-checking the answers received from the parents. The questionnaire was pretested on patients in the dental clinic. Socioeconomic status of the parents was determined (1) according to the occupation of the father, (2) according to the income of the family as reported by the mother, and (3) as estimated by the interviewer after interviewing the mother. The interviewer took into consideration the data obtained from Questions 6 to 14, as well as his personal rating of the family as surmised from the appearance of the home, furnishings, and neighborhood.

Home interviews were obtained from the parents of 279 of the 650 children examined for dental caries incidence. The remaining parents were not interested in cooperating or could not be traced. Data on the 279 children were analyzed by chi square and correlation techniques.[6]

This study, therefore, represents only children whose parents were traceable and cooperative. On the factors we know about, the respondents and total population examined compare as follows:

FACTORS	RESPONDENTS	TOTAL POPULATION EXAMINED
Number of children	279	650
Mean age	3.9	4.2
Median age	4.0	4.0
Age standard deviation	2.06	1.56
Age range	2-6	1-6
def Teeth		
Mean	4.1	3.8
Median	3.0	3.0
Standard deviation	4.06	3.9
Range	0-19	0-19

FINDINGS

Association of Dental Caries With Food Habits, Socioeconomic Status, and Oral Hygiene.—In the present study of 279 children, no association was found (Table IIA) between dental caries (def teeth) and (a) desserts consumed at lunch and/or at dinner, (b) number of times per week cake, pie, ice cream, gelatin, cookies, and/or candy were consumed, (c) number of times per week fresh fruit was consumed, (d) frequency of food consumed between meals, (e) economic level of the parents as estimated by the interviewer, (f) type of father's employment (unemployed, unskilled, skilled, white collar, managerial or owner, professional), (h) income of parents, and (i) number of times per day the child brushed his teeth. Distribution of children according to the number of times canned fruit was consumed in one week as related to def teeth was found to be significant ($r = 0.2$, $P<0.05$).

TABLE IIA

ASSOCIATION OF DENTAL CARIES (DEF TEETH) WITH DIFFERENT FACTORS IN 279 CHILDREN 1 TO 6 YEARS OF AGE

FACTORS	APPROXIMATE CORRELATION COEFFICIENT R*	P†
Dental Caries with:		
1. Desserts consumed at lunch and/or at dinner	No association	—
2. Number of times per week cake, pie, ice cream, gelatin, cookies, and/or candy were consumed	No association	—
3. Number of times per week fresh fruit was consumed	No association	—
4. Frequency of food consumed between meals	No association	—
5. Economic level of the parents (as estimated by the interviewer)	No association	—
6. Type of father's employment (unemployed, unskilled, skilled, white collar, managerial or owner, professional)	No association	—
7. Cultural level of parents (number of magazines subscribed)	No association	—
8. Income of parents (reported by mother)	No association	—
9. Number of times a day the child brushed his teeth	No association	—
10. Number of times a week canned fruit was consumed	$r = 0.2$	$P<0.05$
11. Number of rooms per family	$r = 0.2$	$P<0.05$
12. Number of years of father's education	$r = -0.4$	$P<0.05$
13. Number of years of mother's education	$r = -0.3$	$P<0.05$
14. Age of the child	$r = 0.4$	$P<0.05$

*A short-cut measure of correlation (William A. Spurr[6]).

†Probability of this high an r if no correlation present.

TABLE IIB

ASSOCIATION OF FATHER'S INCOME; TOOTHBRUSHING, TIME AND FREQUENCY; AND AGE OF THE CHILD WITH DIFFERENT FACTORS

FACTORS	APPROXIMATE CORRELATION COEFFICIENT R*	P†
Father's income with:		
1. Number of times per week canned fruit was consumed	No association	—
2. Number of times per week fresh fruit was consumed	No association	—
3. Number of times per week ice cream, cake, pie, gelatin, cookies, and/or candy was consumed	No association	—
4. Education (father)	$r = 0.7$	$P<0.01$
Toothbrushing, time and frequency, with:		
1. Age of the child	No association	—
2. Income of the parents	No association	—
3. Number of decayed teeth (d)	No association	—
4. Number of years of father's education	$r = 0.3$	$P<0.05$
5. Number of years or mother's education	$r = 0.3$	$P<0.05$
Age of the child with:		
1. Number of times per week canned fruit was consumed	No association	—
2. Number of times per week fresh fruit was consumed	No association	—
3. Number of times per week ice cream, cake, pie, gelatin, cookies, and/or candy was consumed	No association	—

*A short-cut measure of correlation (William A. Spurr[6]).

†Probability of this high an r if no correlation present.

Economic status of the parents as determined by the interviewer, income of parents, or the type of employment was not found to be related to dental caries, but the number of rooms per person in the family was associated with incidence of caries ($r = 0.2$, $P<0.05$). Similarly, whereas the cultural level of the parents was not related, the number of years of father's education was rather strongly associated with dental caries ($r = -0.4$, $P<0.05$), and number of years of mother's education also showed some association ($r = -0.3$, $P<0.05$). It will be observed on Table III that parents with high education had fewer children needing dental attention. As expected, age and dental caries were associated ($r = 0.4$, $P<0.05$).

Table IIB shows that no correlation was found between the income of the father and the number of times per week the child consumed (a) ice cream, cake, pie, gelation, cookies, and candies, (b) fresh fruit, and (c) canned fruits. Income of the father was strongly related to the number of years of schooling he had received ($r = 0.7$, $P<0.01$).

TABLE III
ASSOCIATION OF CARIES (DEF TEETH) IN CHILDREN 1 TO 6 YEARS OF AGE WITH NUMBER OF YEARS OF FATHER'S EDUCATION

NO. YEARS EDUCATION	DEF TEETH											TOTAL
	0	1	2	3	4	5	6	7	8	9	10	
0-2	7	3	2	1	1	1	1	2	1	1	1	20
3-4	19	4	8	4	7	9	8	5	7	6	6	83
5-6	12	3	6	3	3	4	3	5	2	3	1	45
7-8	14	7	7	4	11	1	4	3	4	0	2	57
9-10	9	0	2	0	1	0	1	1	1	0	3	18
11 plus	11	2	3	3	5	1	1	0	0	0	1	27
Total	72	17	28	15	28	16	18	16	15	10	14	250*

*29 did not indicate the years of education.
Approximate correlation: $r = 0.42$, $P < 0.05$. See Reference 6.

The time of day when the child brushed his teeth (at rising, before breakfast, after breakfast, after lunch, after dinner, and/or before retiring) as well as the number of times per day he brushed his teeth was not found to be associated with the age of the child, income of the parents, or the number of decayed teeth (d). The children started brushing their teeth at 2 years of age. At 2 to 6 years of age, 6 per cent of the children did not brush their teeth, 52 per cent brushed their teeth once daily, 38 per cent brushed twice daily, and 3 per cent brushed 3 times per day. Of the 52 per cent brushing once daily, two-thirds brushed them before retiring. Of the 38 per cent brushing their teeth twice daily, two-thirds were doing so after breakfast and before retiring. Father's, as well as the mother's, educational level was associated with the number of times per day the child brushed his teeth ($r = 0.3$, $P<0.05$).

No association was found between the age of the child and the number of times per week (a) ice cream, cake, pie, gelatin, cookies, and candies, (b) fresh fruit, and (c) canned fruits were consumed.

SUMMARY

Association of incidence of dental caries with the socioeconomic status of parents was investigated in 279 children. The parent (mother) of the child was interviewed at home by trained interviewers to determine the socioeconomic status and obtain information on the food and oral hygiene habits of the children. A negative association was found between the parent's (father as well as mother) education and dental caries (def teeth). Parent's education was found to be associated with the number of times per day the children brushed their teeth.

Parents' income was not found to be associated with the incidence of dental caries, food, or oral hygiene habits in these 279 children.

We wish to thank Dr. Carl E. Hopkins for his aid in the presentation and supervision of the statistical analysis.

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THE ORAL ADMINISTRATION OF LACTOBACILLUS ACIDOPHILUS INTO THE SYRIAN HAMSTER

I. Oral Population in Relation to Dental Caries

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THROUGH the years numerous investigators[1-7] have reported a close association between oral lactobacilli and dental caries activity in human beings.

Recently a number of workers[8-12] who have worked with either human beings or experimental animals have reported no correlation between oral lactobacilli and dental caries activity.

Orland and Reyniers[13] were able to produce carious lesions in the teeth of germ-free animals when lactobacilli were the only contaminant. A number of investigators[14-18] have attempted to implant lactobacilli in the oral cavities of either human beings or experimental animals in an endeavor to produce dental caries. However, none of these workers demonstrated any evidence for such implantation or for the production of dental caries. Since there is no complete agreement as to the etiological agent of dental caries, information based upon available evidence indicates further investigation of lactobacilli as the suspected agent is warranted.

The purpose of this investigation has been twofold: (a) to determine the possibility of increasing the oral lactobacilli population in experimental animals by routine oral administration of lactobacilli under normal oral conditions and under altered or conductive oral environmental conditions which may promote growth of the test organism, and (b) to determine whether or not the oral lactobacilli population could be correlated with the incidence of dental caries or the gross loss of carious tooth structure.

METHOD

Three litters, totaling 24 animals, of Syrian hamsters from the Hathaway strain were segregated at 23 days of age into 4 groups according to sex and littermates. The 4 groups were differentiated according to diet and were known as regular (R), regular and lactobacilli (RL), cariogenic (C), and cariogenic and lactobacilli (CL). Each animal in all 4 groups received a basal diet of Purina Laboratory Chow which ranged from 5 to 10 Gm. daily, the ration increasing with age of the animals. Animals in the C and CL groups received, in addition to the basal ration, 5 Gm. of chemically pure dextrose daily. Animals in RL and CL groups received orally every day 0.5 to 1.0 c.c. saline solution suspen-

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sion of *L. acidophilus* ATCC No. 4356 at a concentration of 9×10^8 cells per cubic centimeter. The animals in all 4 groups received tap water ad libitum.

Lactobacilli counts of the saliva of all animals were begun when the animals reached 12 weeks of age and were repeated at 2-week intervals until they were 26 weeks old. The saliva samples were procured between 18 and 20 hours following oral inoculation of animals with lactobacilli. Since the technique involved in obtaining and processing saliva samples was a lengthy procedure for any one particular group, only the animals in one group were studied on any one particular day. Therefore, when the counts were made at each 2-week interval, saliva samples were taken for the 4 groups on 4 consecutive days, samples being taken consistently in the same orderly arrangement. Ten

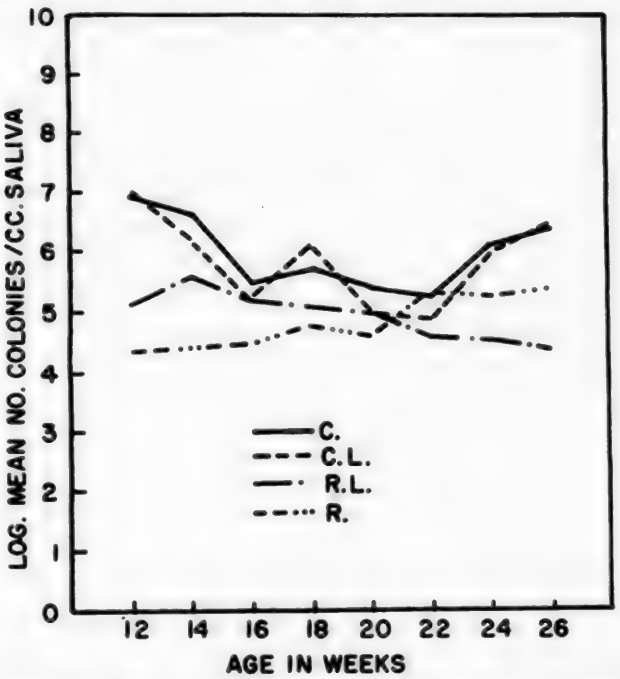

Fig. 1.—Biweekly oral lactobacilli counts plotted according to animal groups.

minutes prior to the obtaining of a saliva sample, each animal received intraperitoneally 0.11 mg. of pilocarpine in 0.04 c.c. of saline solution. Approximately 0.10 c.c. of saliva was obtained from each animal. Saliva was collected aseptically by suction. Saliva-saline solution dilutions of 1-100, 1-500, 1-1000, 1-10,000, 1-100,000 and 1-1,000,000 were prepared for each specimen. Pour plates were prepared in duplicate using 1 c.c. of the respective dilutions given previously and 20 c.c. of Rogosa's medium. The cultures were incubated anaerobically for 72 hours at 37° C. Routinely, the colonies were checked and identified. Colonies verified as lactobacilli were gram-positive nonspore-forming rods which were unable to reduce nitrates and were catalase negative. Counts were made with the aid of a Spencer colony magnifier and a mechanical

counter. At 29 weeks of age, all the animals were killed by inhalation of vaporized ethyl ether. The heads were placed in individual flasks of distilled water and autoclaved at 120° C. for 20 minutes. Soft tissues were then removed from the mandibles and maxillae. The teeth of each animal were examined microscopically for dental caries, gross loss of carious tooth structure, and other defects. Under ×21 magnification, the gross loss of the carious dental tissue for each tooth was determined with the aid of a dental explorer calibrated in one-hundredths of an inch. The volume of the carious area was calculated on the basis of the length, the width, and the depth of the lesion. Such volume here reported as the degree of gross loss of carious tooth structure has been expressed as a whole number. This calculation is the product obtained by multiplying the numerators of 3 common fractions which were determined when the width, length, and depth of each lesion were measured.

TABLE I
A COMPARISON OF THE ORAL LACTOBACILLI COUNTS ACCORDING TO ANIMAL GROUPS

		OBSERVATION PERIODS CORRESPOND TO AGE OF ANIMAL IN WEEKS							
	WEEKS	12	14	16	18	20	22	24	26
Gp. C	No. saliva specimens	6	6	6	6	6	6	6	6
	Mean No. colonies*	9,400	4,600	300	600	280	200	1,300	2,500
	Log. mean No. colonies	6.97	6.66	5.48	5.77	5.44	5.29	6.11	6.39
Gp. CL	No. saliva specimens	6	6	6	6	6	5	5	5
	Mean No. colonies*	9,900	1,700	160	1,300	100	79	1,000	2,800
	Log. mean No. colonies	6.99	6.22	5.19	6.10	5.00	4.89	6.00	6.44
Gp. R	No. saliva specimens	6	6	6	5	5	5	4	4
	Mean No. colonies*	22	27	31	63	41	210	190	250
	Log. mean No. colonies	4.34	4.43	4.49	4.79	4.61	5.32	5.28	5.39
Gp. RL	No. saliva specimens	6	6	6	6	6	6	5	5
	Mean No. colonies*	130	400	170	120	97	40	37	26
	Log. mean No. colonies	5.13	5.60	5.24	5.08	4.98	4.60	4.56	4.41

*Lactobacilli colonies per c.c. saliva × 10^3.

RESULTS

In Table I are shown the biweekly oral lactobacilli counts according to animal groups. The column showing the number of saliva specimens corresponds to the number of animals in the group studied. Four of the original 24 animals escaped from their cages and were lost to the experiment. In Fig. 1, the ordinate represents the logarithm of the mean number of colonies per cubic centimeter of saliva, and the abscissa indicates the age of the animals. Lactobacilli counts for the 4 groups fluctuate from time to time, but each group follows a definite trend.

In Table II, a statistical comparison of oral lactobacilli counts between animals of cariogenic (C) and regular (R) groups at various age periods have been made. For such periods, namely, at 12 and 14 weeks, at 16, 18, and 20 weeks, at 22, 24, and 26 weeks, and for 12 through 26 weeks, the critical ratios 2.18, 3.51, 1.93, and 2.47 are shown, respectively. While such findings have indicated a significant difference between the groups (a critical ratio of above 2.0 is considered significant), they also have indicated that the differences between the 2 groups became less significant as the study continued. Lactobacilli counts of animals of the R group progressively increased from the 12- and 14-week period (24×10^3 colonies per cubic centimeter of saliva) to the 22-, 24-, and 26-week period (220×10^3 colonies per cubic centimeter of saliva). The counts for the animals in the C group, barring certain fluctuations, for the same periods of observation, have decreased from a high of $7{,}000 \times 10^3$ colonies per cubic centimeter of saliva to a low of $1{,}300 \times 10^3$ colonies. Findings in Table II have indicated no significant difference in

TABLE II
A STATISTICAL COMPARISON OF ORAL LACTOBACILLI
BETWEEN C AND R GROUPS AND BETWEEN R AND RL GROUPS

GROUPS	AGE IN WEEKS	NO. SALIVA SPECIMENS	MEAN NO. COL./C.C. $\times 10^3$	S. D. $\times 10^3$	S. E. M. $\times 10^3$	C. R.
C	12, 14	12	7,000	11,160	3,221	2.18
R	12, 14	12	24	24	7	—
C	16, 18, 20	18	390	414	97	3.51
R	16, 18, 20	16	44	68	17	—
C	22, 24, 26	18	1,300	2,397	564	1.93
R	22, 24, 26	13	220	291	80	—
C	12-26	48	2,400	6,373	919	2.47
R	12-26	41	93	189	29	—
R	12, 14	12	24	24	7	2.40
RL	12, 14	12	270	351	101	—
R	16, 18, 20	16	44	68	17	2.95
RL	16, 18, 20	18	130	101	24	—
R	22, 24, 26	13	220	291	80	2.22
RL	22, 24, 26	16	35	36	9	—
R	12-26	41	93	189	29	0.92
RL	12-26	46	130	212	31	—

S. D. = Standard deviation
S.E.M. = Standard error of the mean.
C. R. = Critical ratio.

counts between animals of R (regular) and RL (regular plus lactobacilli) groups based upon total number of observations made during the twelfth through the twenty-sixth weeks. However, there are significant differences in counts as shown for certain periods during the study. Between the twelfth and twentieth weeks of observation, the animals receiving the oral lactobacilli suspension (RL) showed a significantly higher count than did those of the R group. Between the twenty-second and twenty-sixth weeks of observation, the animals of the RL group showed a significantly lower count than those of the R group.

No significant difference was observed in oral lactobacilli counts between the animals of the cariogenic (C) and cariogenic plus lactobacilli (CL) groups at any time during the experiment (Table III). However, significantly higher counts were observed in animals of the C and CL groups in relation to those of the R and RL groups according to any of the 3 comparisons shown.

TABLE III
A STATISTICAL COMPARISON OF ORAL LACTOBACILLI BETWEEN C AND CL, CL AND RL, CL AND R, C AND RL GROUPS

GROUPS	AGE IN WEEKS	NO. SALIVA SPECIMENS	MEAN NO. COL./C.C. SALIVA $\times 10^3$	S. D. $\times 10^3$	S. E. M. $\times 10^3$	C. R.
C	12, 14, 16	18	4,800	9,646	2,273	0.29
CL	12, 14, 16	18	3,900	7,781	1,833	—
C	18, 20, 22	18	360	414	97	0.52
CL	18, 20, 22	17	500	1,074	260	—
C	24, 26	12	1,900	2,768	799	0.01
CL	24, 26	10	1,900	2,038	644	—
C	12-26	48	2,400	6,373	919	0.18
CL	12-26	45	2,200	5,236	780	—
CL	12-26	45	2,200	5,236	780	2.61
RL	12-26	46	130	212	31	—
CL	12-26	45	2,200	5,236	780	2.66
R	12-26	41	93	189	29	—
C	12-26	48	2,400	6,373	919	2.42
RL	12-26	46	130	212	31	—

S. D. = Standard deviation.
S.E.M. = Standard error of the mean.
C. R. = Critical ratio.

Findings in Table IV indicate that the dental caries incidence was greater in the animals of the C group as compared to those of the R group and greater in those of the CL group as compared to the RL group by 28 and 40 per cent, respectively. Likewise, comparing gross loss of carious tooth structure be-

TABLE IV
A COMPARISON OF CARIES INCIDENCE BETWEEN ANIMAL GROUPS AS WELL AS A COMPARISON OF GROSS LOSS OF CARIOUS TOOTH STRUCTURE BETWEEN SUCH GROUPS

GROUPS	MEAN NO. CARIOUS TEETH	PER CENT CHANGE	MEAN D. G. L.*	PER CENT	RESPONSIBLE FACTORS
C	4.16	28.00 I.	7.16	218.2 I.	Glucose
R	3.25	—	2.25	—	
CL	2.80	40.00 I.	4.60	206.6 I.	Glucose
RL	2.00	—	1.50	—	
R	3.25	—	2.25	—	L.a.S.†
RL	2.00	38.47 D.	1.50	33.33 D.	
C	4.16	—	7.16	—	L.a.S.
CL	2.80	32.70 D.	4.60	35.76 D.	
R	3.25	—	2.25	—	Glucose
CL	2.80	13.85 D.	4.60	104.4 I.	L.a.S.

I = Per cent increase; D = per cent decrease.
*D.G.L. = The degree of gross lors of carious tooth structure.
†L.a.S. = Lactobacillus acidophilus in saline solution.

tween C and R groups and between CL and RL groups, the animals on the cariogenic diet showed greater tooth destruction than those on the regular diet by 218.2 and 206.6 per cent, respectively. Table IV also indicates that the dental caries incidence and gross loss of carious tooth structure were less in those groups (RL and CL) which received lactobacilli than in the control groups (R and C). Factors responsible for increasing or decreasing either the caries incidence or the gross loss of carious tooth structure are shown.

The data in Table V indicated no definite correlation between either dental caries incidence or gross loss of carious tooth structure and number of oral lactobacilli between the animals of R and RL groups or between the animals of C and CL groups. It has, however, indicated such a correlation between the animals of R or RL groups and those of C or CL groups.

An additional dental defect known as erosion, of which no mention has been made in any table or chart, was observed from a slight to a marked degree on several teeth of almost all the animals in the RL group. The teeth of the animals of the 3 other groups showed no such defects. Just prior to sacrificing the animals the mean weight of those in the RL group was less when compared to that of the animals for each of the three other groups.

TABLE V
A COMPARISON OF CARIES INCIDENCE AND GROSS LOSS OF CARIOUS TOOTH STRUCTURE WITH NUMBER OF ORAL LACTOBACILLI

GROUPS	NO. OF ANIMALS	MEAN NO. CARIOUS TEETH	MEAN* D.G.L.	MEAN NO. COLONIES/C.C. SALIVA × 10^3 FOR AGE PERIODS SHOWN IN WEEKS			
				12, 14	16, 18, 20	22, 24, 26	12-26
R	4	3.25	2.25	24	44	220	93
RL	5	2.00	1.50	270	130	35	130
C	6	4.16	7.16	7,000	390	1,300	2,400
CL	5	2.80	4.60	5,800	510	1,300	2,200

*D.G.L.—The degree of gross loss of carious tooth structure.

DISCUSSION

The fact that the oral lactobacilli counts of either C or CL groups (Tables I, II, and III) were significantly higher than either R or RL groups confirms the evidence[6, 20-27] for the correlation between oral intake of carbohydrates and the number of lactobacilli in the saliva. Glucose in the diet does predispose conditions for promoting growth of oral lactobacilli. The fact that there was no statistical difference in oral lactobacilli counts between C and CL groups supports the evidence of others[17, 18] who were unable to implant lactobacilli in the mouths of either human beings or experimental animals. Evidently, the successful implantation of bacteria in the mouth depends greatly upon certain environmental factors which in the case of lactobacilli is strongly influenced by the amount of glucose present in saliva and other undetermined factors.

The fact that the oral lactobacilli counts of RL animals (Tables I and II) were much greater than those of R animals during the early weeks of the study does present evidence for the first time that the number of oral lactobacilli can be increased by oral inoculation when the conditions necessary for the growth of the organisms are optimum. That these optimum conditions are not always

present in the mouth is demonstrated by the fact that the oral lactobacilli counts of the RL animals were much less than those of the R animals during the latter weeks of the study.

That the mean oral lactobacilli counts of C and CL animals (Table I) decreased from a high of $9,400 \times 10^3$ and $9,900 \times 10^3$ colonies, respectively, during the first week of observation to a low of $2,500 \times 10^3$ and $2,800 \times 10^3$, respectively, during the last week of observation may be attributed to the decreasing percentage of glucose consumed by the animals as consumption of the basal diet increased. The animals of both the C and CL groups consumed approximately 5 Gm. of glucose per day throughout the study. The basal ration was increased from 5 Gm. daily at the beginning of the study to approximately 10 Gm. daily when the animals became 16 weeks of age. The daily consumption remained constant thereafter until the end of the experiment.

The fact that the mean oral lactobacilli count of the animal in the R group (Table I) continued to rise from a low of 22×10^3 colonies per cubic centimeter of saliva during the first week of observation to a high of 250×10^3 during the last week of observation seems evident. Numerous investigators[2, 4-7, 14, 21, 28-33] have correlated caries activity with increase in oral lactobacilli counts.

While no evidence has been presented for permanently implanting lactobacilli in the oral cavity of animals, under certain conditions it was observed that the oral lactobacilli population of the RL animals (Table I) as compared to that of the control (R) animals continued to be higher for 18 to 20 hours following the administration of such a suspension. Also, in several of the animals receiving the lactobacilli suspension, a marked erosion was observed which was not found to be present on the teeth of the animals in the control group.

The fact that the dental caries incidence in the animals (CL and RL groups) receiving the oral suspension of lactobacilli was less as compared to control groups (C and R) suggests that the lactobacilli may compete for certain growth requirements which are equally necessary for the growth of other microorganisms concerned in the caries process.

The fact that the gross loss of carious tooth structure was greater in the animals receiving the glucose (Table V) may be due to the penetration of the carbohydrate into the tooth substance which would thereby provide a more suitable medium for bacterial growth. Butt,[34] working with freshly extracted teeth and C^{14} sucrose, has shown a diffuse penetration of the enamel by the carbohydrate.

The significantly higher oral lactobacilli counts observed in the animals of the C group compared to those in the R group (Table II C. R. 2.47), and likewise in the CL group, as compared to the RL group (Table III C. R. 2.61) are closely correlated with the greater caries incidence and greater loss of carious tooth structure observed in the C and CL groups as compared to the R and RL groups (Table IV). These findings concur with evidence presented by other investigations.[2, 4-7, 14, 21, 28-33]

The observation where no statistical difference existed in oral lactobacilli counts between R and RL groups (Table II C. R. 0.92) and between C and CL groups (Table III C. R. 0.01) and a marked difference in dental caries activity occurred between each of the groups (Table IV) concurs with similar findings presented by numerous investigators.[9, 10, 35] The results of this study do indicate why there exists today a lack of agreement among investigators concerning the relationship between dental caries activity and the number of oral lactobacilli present in individuals. With respect to the Syrian hamster, which practices coprophagy, it does appear that oral lactobacilli counts cannot be considered wholly reliable as an index of dental caries activity. Such findings are in agreement with those of Helman and Mitchell.[12]

CONCLUSIONS

1. Oral lactobacilli counts of the animals on cariogenic diets (C and CL) were significantly higher than those of animals on regular diets (R and RL), on a basis of total counts made for the entire 15 weeks of observation.

2. The dental caries incidence was greater in the animals of the C group as compared to those of the R group and greater in those of the CL group compared to RL group by 28 and 40 per cent, respectively. Likewise, comparing gross loss of carious tooth structure between C and R groups and between CL and RL groups, the animals on cariogenic diet showed greater tooth destruction than those animals on the regular diet by 218.2 and 206.6 per cent, respectively.

3. Oral lactobacilli counts were not significantly different between animals in the C and CL groups throughout the entire period under observation. Counts were significantly different between animals in R and RL groups for certain periods of time only. On a basis of total counts made during the entire study for these two groups no statistical difference in counts between R and RL animals was observed.

4. Dental caries incidence and gross loss of carious tooth structure was less in RL animals as compared to R animals by 38.47 and 33.33 per cent, respectively. Dental caries incidence and gross loss of carious tooth structure was less in CL animals as compared to C animals by 32.70 and 35.76 per cent, respectively.

5. While the oral lactobacilli population was shown to be closely associated with dental caries activity, there was observed no absolute correlation between it and the incidence of dental caries or the gross loss of tooth structure.

6. While no evidence has been presented for permanently implanting lactobacilli in the oral cavity, the oral administration of *L. acidophilus* under certain conditions resulted in an increase in the oral lactobacilli population. Dental erosion was observed in several of the animals which received the lactobacilli orally. Animals not receiving the lactobacilli showed no such evidence.

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AMMONIA PRODUCTION IN SALIVA. I

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IN A previous paper,[1] we reported on the presence of an ammonia-producing mechanism in saliva which, on incubation of the saliva for 24 hours at 37° C., produced 5 to 10 times the amount of ammonia originally present. The addition of glucose (2.5 per cent final concentration) to the incubating mixture inhibited ammonia production. The addition of urea (1.5 per cent final concentration) enhanced ammonia production, showing the presence of an active urease in saliva. The addition of both glucose and urea stimulated the production of ammonia beyond the amount produced when urea alone was added.

In a second paper,[2] it was shown that there was no relationship between the activity of the ammonia-producing mechanism and caries activity as measured by lactobacillus counts. The urease activity of the salivas studied also showed no relationship to caries activity. The stimulating effect of glucose upon ammonia production in the presence of urea was observed in all the salivas studied.

In an attempt to shed further light on the mechanism of glucose inhibition of ammonia production, on the one hand, and glucose stimulation of ammonia production in the presence of urea, on the other, the following experiments were performed.

EXPERIMENTAL

1. *The relationship between pH and ammonia production in saliva* is shown in Table I. The Folin-Bell[3] method was used to determine ammonia. A veronal-acetate buffer was used to control the pH.

TABLE I
THE RELATIONSHIP BETWEEN pH AND AMMONIA PRODUCTION IN SALIVA

TUBE NO.	pH		AMMONIA CONTENT (MG. %)
	AT START	AT 24 HR.	
1	4.42	4.60	10.9
2	5.50	5.79	14.3
3	6.62	6.81	31.4
4	7.98	7.92	22.5
5	8.46	8.42	9.5

These results indicate that the optimum pH range for ammonia production is 6.5 to 7.0.

2. *The inhibitory effect of glucose on ammonia production* might be due to the fact that the added glucose forms a substrate for acid-producing bacteria,

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which would produce acid and thus lower the pH below the optimum for ammonia production. To test this theory, a series of buffered tubes (pH 7.0) were incubated with and without glucose. The results obtained are shown in Table II.

TABLE II
THE EFFECT OF GLUCOSE ON AMMONIA PRODUCTION BY SALIVA BUFFERED AT 7.0

TUBE NO.	GLUCOSE (ML. 20%)	SALIVA (ML.)	pH AFTER 18 HR.	NH_3 CONTENT (MG. %)
1	1	2	6.70	None
2	0	2	7.00	16.0
3	1	2	6.75	Trace
4	0	2	7.00	17.5

Since glucose inhibited ammonia production even when the tubes were buffered at pH 7.0, the lowering of the pH is not the only factor in the inhibition of ammonia production by glucose. The buffer used in these experiments was found to have a slight inhibitory effect on ammonia production, which accounts for the somewhat lower ammonia values in this experiment.

3. *The stimulating effect of glucose on ammonia production by saliva-urea mixtures* could be simply a pH effect. The acid production by bacterial enzymes acting on the glucose could keep the pH at the optimum for ammonia production (7.0) for a longer time, thus producing more ammonia.

To shed light on this question, the ammonia production with or without added glucose by buffered urea-saliva solutions was observed. The results are shown in Table III.

TABLE III
THE EFFECT OF GLUCOSE ON AMMONIA PRODUCTION BY UREA-SALIVA MIXTURES BUFFERED AT pH 7.0

TUBE NO.	SALIVA (ML.)	UREA (ML. 20%)	GLUCOSE (ML. 20%)	pH AFTER 18 HR.	NH_3 CONTENT AFTER 18 HR.
1	2	1	1	7.20	92.5
2	2	1	0	7.70	85.0
3	2	1	1	7.25	92.5
4	2	1	0	7.75	87.0

These results indicate that the stimulating effect of glucose could be attributed to acid keeping the pH near the optimum for ammonia production. Glucose in buffered solutions showed no stimulating effect on ammonia production.

DISCUSSION AND CONCLUSIONS

A certain amount of disagreement seems to exist in the literature as to whether ammonia in saliva is related to dental caries. Saliva contains bacterial enzyme systems capable of producing acid which could decalcify enamel, and other bacterial enzyme systems capable of producing ammonia. Our work has shown that the addition of glucose to saliva inhibits ammonia production and that this inhibition is not altogether attributable to the acid produced, lowering the pH beyond the limit for ammonia production. Glucose per se in buffered solutions inhibits ammonia production.

Glucose, when added to urea-saliva mixtures, enhances ammonia production, and in this case pH seems to be a decisive factor. In buffered solutions (pH 7.0), the stimulating effect of glucose on ammonia production is no longer observed.

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THE EFFECTS OF CASTRATION, SEX HORMONES, AND DESALIVATION ON DENTAL CARIES IN THE RAT

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INITIAL experiments in these laboratories have indicated that desalivation in the immature rat resulted in additional unexplained biologic changes, other than an increase in caries.[1-3] Notable among these were changes in the adrenal glands, testes, and uterus,[3] and an apparent altered skeletal fluorine storage.[4] Food consumption, however, in desalivated animals is an important consideration in the estimation of the amount of fluorine stored in the skeleton.[4] The relationship between the salivary glands and the endocrines, and between dental caries and the endocrines, is not well defined. Since castration results in characteristic changes in the salivary glands, and concomitant reductions in dental caries are found in male rats, it appears that some relationship between the gonads, the salivary tissues, and the incidence of dental caries in the rat exists. The most striking change in the salivary gland, following castration, is in the size and number of the granular tubules of the submaxillary gland,[2, 5] but whether or not this represents an altered salivary function is not yet clear.

In addition, castration produced changes in the rat adrenal gland,[6] and the removal of the salivary glands produced alterations in the zona glomerulosa of the adrenal gland, as well as some weight changes.[3] Although these weight changes were subsequently shown to depend primarily upon the nutritional status of the desalivated animals, the testicular and uterine weight alterations were not completely explained on this basis. The purpose of this report then is twofold: first, to present data concerning the effect of sex hormones on dental caries and fluorine storage and to extend the previous work over the effects of castration and desalivation on dental caries and fluorine storage; and second, to report the results of a sham desalivation operation on dental caries, food consumption, the endocrine organs, and fluorine storage, as well as to consider body and organ weight relationships in desalivated and unoperated animals.

EXPERIMENTAL

Approximately 100 weanling Sprague-Dawley strain rats were divided equally as to sex and weight into 3 experimental groups and designated as Series I. One group was desalivated by a procedure already reported,[4] another was sham-desalivated, and the third group was unoperated and served as controls. The animals received our cariogenic diet at 30 days of age.[7] The duration of the experiment was 140 days, during which time all of the animals

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had tap distilled water and diet available ad libitum. They were housed in raised-screen cages in a temperature-controlled room. At the termination of the experimental period, they were sacrificed by ether inhalation, the heads removed for dental caries evaluation, and the femurs removed for calcium, phosphorus, and fluorine analysis. The adrenals, uterus, and testis were weighed on an analytic balance and fixed in Bouin's picric acid fixative for histologic examination. The thyroid, pituitary, and salivary glands were also fixed for histologic study. The daily food consumption was measured in order to evaluate the organ weight changes and fluorine storage in relation to the amount of food ingested. Twice each week, the food which had spilled was collected, weighed, and allowed for in calculating the animal's daily food consumption. Water consumption was also measured each day.

Series II was composed of approximately 140 weanling rats divided into 5 experimental groups. Group I was castrated at 30 days of age, while Group II was castrated at 60 days of age. Group III was both castrated and desalivated at 30 days of age, and a fourth group was operated identically to Group III but, in addition, received the respective androgenic or estrogenic supplements. The males had intramuscular injections every other day of 400 μg of testosterone propionate,* and the females received 200 μg of estradiol dipropionate† subcutaneously, every fourth day. Group V was unoperated and served as a control. After 140 days, the animals were sacrificed by ether inhalation and the various organs, heads, and femurs removed and analyzed as previously described.

DATA AND DISCUSSION

Table I indicates the weight gains and daily food and water consumption, as well as the dental caries experience and fluorine storage in the Series I animals. The desalivated rats failed to gain as much weight as the control or sham-operated animals. Dale[8] and Shaw and Weisberger[9] also have reported this finding. An examination of the daily amounts of food consumed by these animals suggests that this failure to gain weight is attributable to a decreased food intake. This, however, is not the complete answer since the desalivated females ate more food than the sham-operated females and still failed to gain as much weight. The water consumption data are also interesting in that the desalivated animals drank considerably less water, as compared to the control animals. The reason for this lowered water intake is not yet clear, but it appears to be correlated in some manner to the decreased food consumption.

Desalivation also resulted in a pronounced increase in dental caries which is in accord with previous findings.[4, 10-13] Although the sham-operated males had a slightly lower caries score than their controls, this reduction was not significant. Both the desalivated males and females stored less fluorine than the respective control animals. These results are in accord with previous findings,[4, 13] but this is most likely attributable to the lower food consumption

*Perandren (Ciba).
†Ovocylin (Ciba).

TABLE I

EFFECT OF DESALIVATION ON FOOD AND WATER CONSUMPTION, DENTAL CARIES, AND FLUORINE STORAGE. SERIES I

	FINAL BODY WEIGHT (GM.)	WEIGHT GAIN (GM.)	FOOD CONSUMED (GM./DAY)	WATER CONSUMED (ML./DAY)	DENTAL CARIES (NO. LESIONS)	% CHANGE	SKELETAL ANALYSIS (ASHED FEMUR)				
							WEIGHT (GM.)	FLUORINE CONCENTRATION (μG./GM.)	TOTAL FLUORINE (MG.)	CALCIUM (MG. %)	PHOSPHORUS (MG. %)
					Males						
Control	358	300	14.7	22.3	7.6 ± 1.9*	—	0.3568	42.6	0.016	41.8	19.5
Sham-operated	339	286	14.5	21.5	6.4 ± 2.3	-15.8	0.3499	34.1	0.012	41.5	19.0
Desalivated	305	252	13.4	17.1	11.8 ± 1.3	+55.4	0.3483	24.1	0.009	43.7	18.8
					Females						
Control	223	173	11.5	18.4	6.7 ± 2.5	—	0.2759	49.0	0.014	41.4	19.5
Sham-operated	223	173	11.2	19.1	6.5 ± 2.5	-3.0	0.2739	35.1	0.014	42.5	19.2
Desalivated	207	157	11.5	15.3	12.6 ± 1.5	+88.0	0.2569	31.0	0.008	40.8	18.7

*Standard deviation.

TABLE II

EFFECT OF CASTRATION, DESALIVATION, AND SEX HORMONES UPON DENTAL CARIES INCIDENCE AND FLUORINE STORAGE. SERIES II

	FINAL BODY WEIGHT (GM.)	WEIGHT GAIN (GM.)	NUMBER OF ANIMALS	DENTAL CARIES (NO. LESIONS)	% CHANGE	SKELETAL ANALYSIS (ASHED FEMUR)				
						WEIGHT (GM.)	FLUORINE CONCENTRATION (μG./GM.)	TOTAL FLUORINE (MG.)	CALCIUM (MG. %)	PHOSPHORUS (MG. %)
				Males						
Control	298	229	14	6.8 ± 2.2*	—	0.3007	77.6	0.024	40.9	20.8
Castrate at 30 days	266	198	14	4.6 ± 2.0	-32	0.2651	95.8	0.026	41.1	20.0
Castrate and desalivate	219	155	11	8.8 ± 1.6	+29	0.2453	76.9	0.019	40.4	19.8
Castrate and desalivate plus testosterone	238	175	9	10.3 ± 2.3	+51	0.2627	55.9	0.014	40.9	19.4
Castrate at 60 days	249	166	15	5.3 ± 2.0	-22	0.2589	54.8	0.013	42.1	20.1
				Females						
Control	206	132	11	6.6 ± 2.6	—	0.2692	93.8	0.025	41.3	19.8
Castrate at 30 days	244	168	13	4.8 ± 2.1	-27	0.2449	82.0	0.020	40.8	19.4
Castrate and desalivate	195	139	14	9.3 ± 2.2	+41	0.2153	72.0	0.015	40.8	19.5
Castrate and desalivate plus estradiol	153	88	12	10.5 ± 2.4	+59	0.2231	71.2	0.016	40.8	19.5
Castrate at 60 days	236	160	13	6.5 ± 3.1	-2	0.2545	52.2	0.013	41.1	20.0

*Standard deviation.

in the operated animals, since paired-feeding techniques have indicated that there is very little difference in fluorine storage in desalivated and control animals when they ingest equal amounts of food.[4]

The dental caries and fluorine storage data from the Series II animals are found in Table II. Castration at 30 days of age resulted in a significant reduction in caries in the males, while castration at 60 days produced no statistically significant effect in either sex. This result serves to emphasize the fact that the age of the animal at the time of operation is an important factor, and one which may explain the somewhat variable caries response in the ovariectomized rat.[1, 10] However, it has been shown that the age at which the animal is placed on the cariogenic diet is also an important variable.[14]

In those rats which were both castrated and desalivated, there was an increase in caries which was in accord with previous results.[4] Nevertheless, by comparing the percentage of increase in dental caries of these animals to that of the desalivated animals in Table I, it is apparent that castration still maintained some effectiveness in reducing the caries incidence in desalivated animals. Apparently, the caries-reducing effect afforded by castration was not completely eliminated by the removal of salivary tissue. The antithesis of this reasoning was confirmed by the much higher caries incidence in the castrated and desalivated animals receiving hormone supplements.

In Table III, the organ weights of both series of animals are presented. Testis and uterine weight changes were manifested again in the desalivated animals, and the decrease in testis weight in the desalivate male was significant at the 1 per cent level of confidence. In previous experiments from this

TABLE III
THE EFFECT OF CASTRATION, DESALIVATION, AND SEX HORMONES ON THE ADRENAL, UTERUS, AND TESTIS WEIGHT. SERIES I AND II

	SEX	ADRENAL WEIGHT (MG./100 GM. BODY WT.)	UTERUS WEIGHT (MG./100 GM. BODY WT.)	TESTIS WEIGHT (MG./100 GM. BODY WT.)
Series I				
Control	M	13.3 ± 2.8*	—	—
	F	25.7 ± 3.4	183.9 ± 37.4	—
Castrated at 30 days	M	15.7 ± 1.8	—	—
	F	18.6 ± 2.6	12.1 ± 2.0	—
Castrated and desalivated	M	17.5 ± 3.2	—	—
	F	20.7 ± 3.1	13.3 ± 1.8	—
Castrated and desalivated + testosterone	M	15.0 ± 2.7	—	—
Castrated and desalivated + estradiol	F	33.0 ± 5.8	207.5 ± 30.7	—
Castrated at 60 days	M	16.1 ± 2.5	—	—
	F	18.2 ± 2.2	20.7 ± 2.8	—
Series II				
Control	M	10.8 ± 2.4	—	784.1 ± 151
	F	19.6 ± 2.3	143.6 ± 35.2	—
Sham-operated	M	10.8 ± 2.8	—	766.0 ± 116
	F	19.3 ± 2.8	138.9 ± 38.6	—
Desalivated	M	11.2 ± 1.9	—	707.0 ± 148
	F	21.4 ± 3.4	167.2 ± 58.1	—

*Standard deviation.

laboratory, there has been found both a significant increase and decrease in the testis weight of desalivated animals. Since the testis weight response to desalivation has been so variable, these results would seem to indicate that nutritional factors are involved, rather than some specific hormonal imbalance.

Desalivation resulted in an increased uterine weight, which also has been found previously, but the weight variation in this group makes the difference of doubtful significance. No significant changes in the adrenal weights of the desalivated animals were observed. However, castration resulted in an increased adrenal weight in the male and a decreased adrenal weight in the female, which confirms the previous observations of other investigators.[6] It is interesting to note that castration at either 30 or 60 days of age produced adrenal changes of similar magnitude in both the males and females. In the animals receiving hormone supplements, testosterone injections resulted in a reduced adrenal weight, whereas the administration of estradiol increased it quite markedly. This result is in accord with the observations of Ito[15] and Rennels.[16]

SUMMARY

Orchiectomy at 30 days of age resulted in a significant reduction in dental caries, while ovariectomy at this age produced only a tendency toward reduction. Castration at 60 days of age had no significant effect on dental caries in either sex. In the animals which were both castrated and desalivated, gonadectomy was still effective in reducing the incidence of dental decay. Hormone injections into these animals resulted in a dental caries score of approximately the same magnitude as that found in the animals which were desalivated.

Castration resulted in an increased adrenal weight in the male and a decreased adrenal weight in the female, while desalivation had no significant effect. Desalivation resulted in a significant decrease in testis weight, while the effect on the weight of the uterus was of doubtful significance.

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FLUORINE STORAGE IN RATS RECEIVING DIFFERENT FLUORIDES IN THE DRINKING WATER AT VARYING pH

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ANY explanation of the usefulness of fluorine as an anticariogenic agent should account for the ultimate fate of fluorine in the organism. Previous work[1-3] has indicated that a positive correlation between fluoride storage in the skeleton and the incidence of dental caries is not tenable, since some fluorides reduce the dental caries experience more than others, yet store considerably less fluorine. Several factors which could affect the metabolism of the element may account for this apparent discrepancy, such as a difference in the absorption of various fluorides by the gut, the effect of the cation accompanying the fluorine, solubility of the fluoride, or the pH of the fluoridized drinking water. The effect of fluorine storage in the skeleton of the rat ingesting fluoridized water solutions ad libitum at two different pH's has been studied,[4] and these data indicate that the storage in the skeleton is independent of pH. However, stannous fluoride was more effective in reducing dental caries in the rat, yet stored considerably less fluorine in the skeleton. The decreased fluorine storage could be due to the ingestion of less fluorine by the animals and not related to differences in metabolic availability of the compounds. Obviously, such considerations are only relative to fluorine storage and in no way explain one fluoride's apparent superiority in reducing dental caries over others. The purpose of this paper, then, is to study the effects of 3 different fluorides administered to rats in the drinking water at different pH's and under controlled conditions of water intake on fluorine storage in the skeleton.

Data furnished by measuring the amount of fluorine in the entire skeleton, using stomach-tube techniques in which the rat is given identical amounts of different fluorides, are also presented.

EXPERIMENTAL

Approximately 225 rats of the Sprague-Dawley strain were divided into 4 experimental groups and placed on a cariogenic corn diet[5] when 30 days old. Each of the groups receiving fluoride supplements had them administered in the drinking water at a fluorine concentration of 20 μg per milliliter. In addition, each of the fluoride groups, and the control group which received no added fluorine, had their drinking water adjusted to pH 2, 4, and 6 by the addition of either sodium hydroxide or hydrochloric acid. After 12 hours,

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the pH of the various solutions was rechecked. The values never varied over one-half of a pH unit. All the 4 experimental groups had their drinking water restricted in such a manner as to assure an equal consumption for all, by determining which group drank the least amount, and providing the others an identical amount. The pH 2 stannous chlorofluoride group drank the smallest volume each day, so each group received the same amount of fluoridized drinking water per day as was consumed by this group. In every instance, all the solutions were given to the animals at the same time each day. All animals were housed in metal cages with raised screen bottoms in a temperature-controlled room. After the experimental period of 140 days, the animals were sacrificed by ether inhalation, the heads were removed for caries evaluation,[5] and the femurs removed for fluorine analysis.[3]

The procedure for ashing whole animals for carcass analysis was as follows: the animal was sacrificed and the entire skin removed in order to prevent fluorine contamination from external sources; the femurs were analyzed for fluorine; the remainder of the carcass was placed in a 3½-inch platinum dish and charred over a meeker burner; the charred mass was transferred to a 3-inch silica dish and placed in the muffle furnace at 550° to 600° C. for 6 to 8 hours. When the ash was cooled, it was weighed, pulverized, and then mixed, using a flattened glass rod. A sample was accurately weighed and placed in the fluorine stills for analysis. An attempt was made to determine the amount of fluorine lost during charring by drawing the fumes from the ashing procedures through a water-cooled condenser, and a flask containing a solution of calcium oxide was arranged so that all of the condensate passed through the calcium oxide. The contents of the flask were then dried and analyzed for fluorine. The results indicated a loss of approximately 1 per cent of the total fluorine in the animal carcass.

DATA AND DISCUSSION

The dental caries experience in all 4 groups was affected by the pH of the drinking water. In each instance, the animals receiving the pH 2 fluoridized drinking water as well as the nonfluoride control pH 2 water had a higher degree of protection than either of the other higher pH solutions (Table I). The pronounced caries reduction at pH 2 with sodium fluoride was decisively lost when the pH of the fluoridized drinking water was increased to 4 or 6. Similar, but to a smaller degree, was the loss in effectiveness when the pH of the drinking water in the tin fluoride groups was increased to 4 or 6. The degree of tooth destruction in the various groups appeared to indicate that all the fluoride groups had a higher degree of enamel erosion than the nonfluoridized control group.

The actual amount of dental caries in every pH 2 group was difficult to determine, due primarily to the fact that the pronounced erosion affected the observation of the normally observed caries pattern to the extent that incipient caries had a decidedly different appearance than in the higher pH groups, or in those animals of previous studies. The apparent caries reduction

in the low pH control group is interesting. In most instances, it was the animals receiving the fluoridized drinking water which presented the most difficulty in determining the presence of a small lesion. These facts may explain the observation (Table I) that sodium fluoride was more effective than stannous fluoride at pH 2, since previous work has always indicated the opposite effect, regardless of the pH of the solution.[4, 6] At the higher pH values, both stannous fluoride and stannous chlorofluoride were about 50 per cent more effective than sodium fluoride at the same pH in reducing the caries experience. These observations corroborate previous in vitro data[6, 7] and in vivo data,[1-3, 5] as well as clinical dental caries studies in children.[8, 9]

The low pH sodium fluoride group seemed to have the greatest degree of erosion, the stannous chlorofluoride group less than the stannous fluoride, and the water control at pH 2 the least destruction. Furthermore, almost 80 to 90 per cent of the enamel erosion occurred in the mandibular teeth.

TABLE I

GROUP	NUMBER OF LESIONS	PER CENT REDUCTION	NUMBER OF LESIONS	PER CENT REDUCTION
Sodium fluoride	1.9	63	1.9	63
	6.1	10	6.1	10
	6.2	11	6.2	—
Stannous fluoride	2.5	51	2.5	51
	4.9	28	4.9	28
	5.6	20	5.6	6
Stannous chlorofluoride	2.4	53	2.4	53
	6.0	32	4.4	35
	4.7	28	4.7	22
Distilled water	5.1	—	5.1	—
	6.8	—	6.8	—
	6.0	—	6.0	—

The effect of the various pH drinking water solutions on fluorine storage in the skeleton is important in light of previously published data.[1-3] In all previous studies, it has been found that when sodium fluoride and stannous fluoride are fed under identical fluorine concentrations, less fluorine is stored in the skeleton by stannous fluoride than with sodium fluoride. Similarly, stannous fluoride is less effective in bleaching the incisors of rats or in causing mottled enamel than sodium fluoride.[10] However, the data from these studies indicate (Table I) that stannous fluoride and sodium fluoride store almost identical amounts of fluorine in the skeleton. Since in this study all the various groups ingested essentially the same quantity of fluoridized drinking water, it seems reasonable that they should store similar amounts of fluorine provided both are equally absorbed from the gut. The now apparent reason for previous studies failing to demonstrate this may be that since the stannous fluoride drinking water solutions are always more acidic than corresponding sodium fluoride solutions, the animals on tin compounds could have ingested less fluorine. However, assuming that previous stannous fluoride groups drank less fluoridized water than similar groups receiving an identical con-

centration of sodium fluoride, yet produced a caries reduction considerably greater than animals receiving sodium fluoride, it would indicate that if the total amounts consumed had been similar, the stannous fluoride animals might have shown an even greater caries reduction as compared to sodium fluoride. An explanation for the apparent difference in storage of fluorine in the stannous chlorofluoride group is not possible at this time.

TABLE II
AMOUNT OF FLUORINE STORED BY DIFFERENT FLUORIDES IN THE FEMUR AND ENTIRE BODY OF THE RAT AFTER RECEIVING 2.0 MG. OF FLUORINE PER DAY FOR 14 DAYS BY STOMACH TUBE

FLUORIDE	F CONC. (μG/GM.)		TOTAL F (MG.)		TOTAL BODY (MG.)	PER CENT F STORED OF TOTAL INGESTED
	FEMUR	BODY	FEMUR	BODY		
NaF	2,714	2,244	0.532	12.78	13.84	53
Na_2PO_3F*	3,027	2,118	0.572	11.50	12.65	47
SnClF	2,176	2,117	0.454	12.65	13.56	51
SnF_2	2,836	2,018	0.540	11.28	12.36	46

*This was kindly made available to us by Dr. Wayne White of the Ozark-Mahoning Co.

Table II indicates the amount of fluorine stored in the entire rat carcass after receiving, by stomach tube, 2.0 mg. of the different fluorides per day for 14 days. All fluorides were made to identical concentrations. Analytic analysis indicated all solutions contained 1,000 ± 2.0 μg F per milliliter. By use of the stomach tube technique, exactly 2.0 ml. of the respective fluoride stock solutions was given to the rats. A total of 4 rats was used for each of the 4 different fluorides. The fluoride supplement was given at the same time and under identical conditions each day. Special considerations were given as to the feeding habits of the animals such that the fluoride was always administered when the stomach was empty.

The data clearly indicate that when identical quantities of fluorine are given to the rat similar amounts are found in the skeleton. However, there appears to be somewhat less total fluorine in the rat carcass of those animals receiving either the sodium monofluorophosphate or stannous fluoride. When the total amount of fluorine ingested in the drinking water (F = 0.1 ppm) and diet (F = 0.3 ppm) is added to the total amount administered by stomach tube and the percentage stored to percentage ingested, calculated from these data, it is interesting to note again that it is the monofluorophosphate and the stannous fluoride groups in which the least amount of fluorine is absorbed. Further, it is important to note that only approximately one-half of the total amount of ingested fluorine is retained by the rat, the balance being excreted by the urine or other routes, or is not absorbed from the gut.

SUMMARY

The pH of fluoridized drinking water determines to a major degree the effectiveness of stannous fluoride or stannous chlorofluoride in reducing the incidence of dental caries in the rat. As the pH is increased from 2 to 4, the

effectiveness is decreased by approximately 50 per cent. Stannous chlorofluoride appears superior to both stannous fluoride and sodium fluoride, even at higher pH values.

When identical amounts of fluorine were given, similar amounts of fluorine were stored in the femurs. In rats given fluorine by means of a stomach tube, only one-half the ingested fluoride was stored in the carcass. Stannous fluoride and sodium monofluorophosphate appeared to cause the storage of less fluorine than stannous chlorofluoride or sodium fluoride, when the compounds were given by stomach tube in aqueous solution.

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METABOLISM OF SMALL CONCENTRATIONS OF FLUORINE IN THE ALBINO RAT

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IT HAS been repeatedly demonstrated that bones and teeth have an affinity for fluorine. Its storage in the skeleton is suspected to be due to the replacement or exchange of fluorine for hydroxyl or bicarbonate groups in the apatite structure, but as yet there is not complete agreement among investigators regarding this mechanism. Nonetheless, fluorine accumulates in the skeleton whether present in the food or water, the final amount stored from the 2 sources differing only in degree. This is important since accumulation of fluorine may result in adverse effects if the level in the diet or water becomes excessive. Such factors as age, previous exposure, the chemical nature of the fluoride compound, the presence or absence of various inorganic ions, the climate in which one lives, and the over-all nutrition of those ingesting the fluoride supplement are a few of the variables which must be considered before any definite conclusions may be made regarding its pharmacologic or toxicologic effects.

Various attempts have been made previously to determine the metabolic turnover of fluorine in the rat as an insight into the metabolism of fluorine by the human being. The experiments of Zipkin and McClure,[1] Savachuck and Armstrong,[2] Miller and Phillips,[3] and Glock, Lowater, and Murray[4] were conducted by administering fluoride to the animals in concentrations ranging from 20 to 450 ppm F for periods ranging from 6 to 40 weeks. After withdrawal of the fluorine supplement from the diet, the animals were sacrificed at various intervals and fluorine analysis performed on the femurs, humeri, or other parts of the skeletal system in order to evaluate the amount of fluorine stored in the animal. Although this method has furnished information concerning the turnover of fluorine in the skeleton, it would seem more desirable to study the metabolism of fluorine by the entire rat. Furthermore, a knowledge of the amount of fluorine found in the feces and excreted in the urine is necessary before it is possible to ascertain the degree of availability and storage rates of fluorine in various compounds when present in the food and drinking water under different conditions. The purpose of this paper, then, is to present preliminary data concerning the techniques of estimating the metabolism of small concentrations of fluorine in the rat by determining the amount of fluorine found in the feces, excreted in the urine, and stored in the entire rat after the administration of known amounts of sodium fluoride. The effect on fluorine

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metabolism was also studied in rats which had had previous exposure to fluorine, in an attempt to determine whether less fluorine is stored in the skeleton if the rat has previously ingested moderate levels of fluorine.

PROCEDURE

All animals used in these studies were housed in individual, round, stainless steel cages, approximately 7½ inches in diameter, having a 2-mesh stainless steel floor. The food cup was securely attached to a 2-inch stainless steel tube approximately 5 inches long with a baffle so placed as to minimize spillage, while allowing the animal free access to the food. The diet was weighed into the food cup and reweighed at the end of each 3-day period to determine the amount of food ingested. A measured amount of drinking water was placed in a 50 ml. graduated cylinder which had the upper portion removed below the pouring spout in order to allow the use of a rubber stopper for attaching the drinking tube. The drinking tube was placed over a thistle tube attached to a covered beaker in order to collect any spilled water. Any water collected in the beaker was measured at the termination of each 24-hour period and allowed for in estimating the total water consumption. These procedures allowed for fairly accurate estimations of the amount of food and water consumed by each animal.

The cage was placed over a round Pyrex dish containing 2 8-inch circles of filter paper. At the end of each 3-day period, the filter papers were removed and replaced with new papers. The feces were previously removed from the filter paper and ashed with exactly 1.00 Gm. of low-fluorine calcium oxide.* The urine, absorbed by the filter paper, was prepared for fluorine analysis in a similar manner. The ashed samples were steam distilled at 135° to 138° C. from perchloric acid and silver perchlorate. The distilled samples were then titrated with thorium nitrate, using alizarin red as indicator, similar to the method described by Smith and Gardner.[5]

The blanks for the calcium oxide and filter paper plus calcium oxide required 1.37 and 3.17 ml. thorium nitrate, respectively. Known amounts of fluorine were added to the filter paper, ashed, and analyzed by this procedure. The average deviation from the known amounts of fluorine recovered was ± 2.0 μg F. The average deviation for 5 determinations when 10.0 μg F was added to the filter papers was ± 0.6 μg F. This approximates the accuracy of the method itself when the aqueous fluoride is added directly to the still. Therefore, it was concluded that this method was satisfactory for the determination of fluorine in urine absorbed by the filter paper. A preliminary study of 12 animals gave satisfactory results.

Two groups of albino rats of the Sprague-Dawley strain were used in these experiments. The animals in Group A received distilled water and a stock corn diet until they were 28 days of age. The animals in Group B received the same stock corn diet, but their drinking water contained 50 μg F per milliliter (as sodium fluoride) from the time they were 14 days of age until they were 28 days old. When both groups were 28 days old, each was

*Obtained from the Fisher Scientific Co., Pittsburgh.

divided into 2 additional groups. One half of each original group received distilled water and the other half received 5 μg F per milliliter as sodium fluoride. This division of groups now resulted in 4 groups completing the metabolism studies. One was designated Group A (no previous added fluorine), which received distilled water, and a second Group A′, which received 5 μg F per milliliter. The third was Group B (50 μg F per milliliter for 14 days previous to the metabolism study), which received distilled water, and the fourth was Group B′ which received 5 μg F per milliliter. All of the animals remained on this regimen for 80 additional days. The stock corn diet contained 0.56 μg F per gram, the distilled water contained 0.06 μg F per milliliter, and the sodium fluoride solution used in all the metabolism studies contained 5.06 μg F per milliliter. All fluorine analyses of the food and water were obtained by repeated chemical analysis.

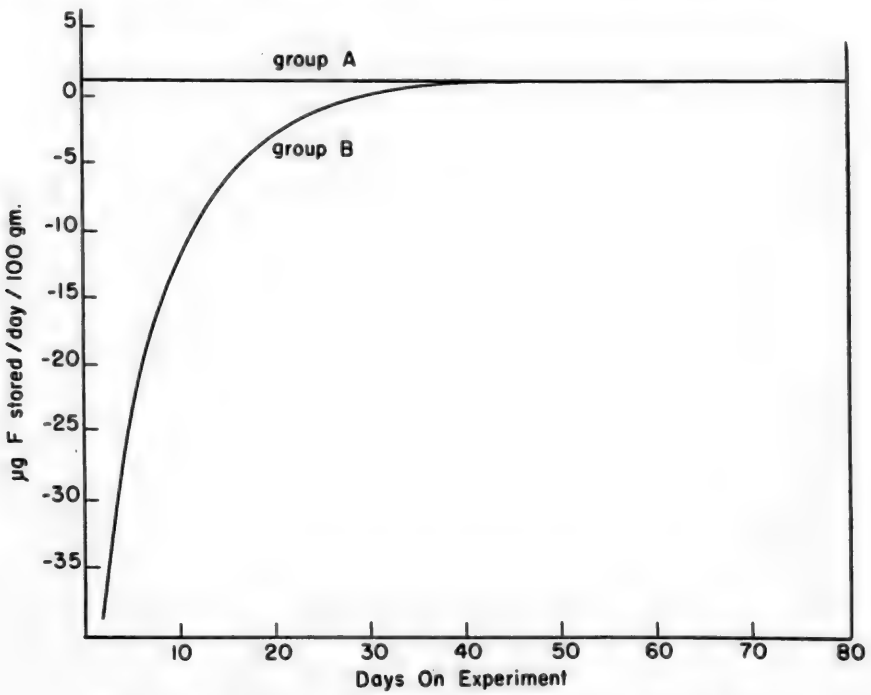

Fig. 1.—Rate of storage of fluorine by animals drinking distilled water.

RESULTS AND DISCUSSION

The major portion of the results of these experiments is represented graphically. Fig. 1 represents the rate of fluorine storage by the two groups of animals which received distilled water after 28 days of age. The Group A animals which received only distilled water and the stock corn diet retained fluorine at the more or less constant rate of 1.5 ± 0.5 μg F per day per 100 grams body weight. This accumulation of fluorine in the skeleton undoubtedly re-

sulted from the small amount of fluorine in the diet and in the distilled water. However, the Group B animals which had previously received fluorine, but which were placed on distilled water at 28 days of age, showed a loss of fluorine during the first 3 to 4 weeks of the metabolism study. After about 40 days, they began to retain fluorine at about the same rate as the Group A (control) animals (Fig. 6). These data appear to indicate that the fluorine retained by the skeleton is not permanently fixed, but is dynamic to the extent that a portion of it is catabolically removed when the level of ingested fluorine is decreased.

Fig. 2 represents the rate of excretion of fluorine in the urine by the same animals represented in Fig. 1. The amount of fluorine in the urine of the

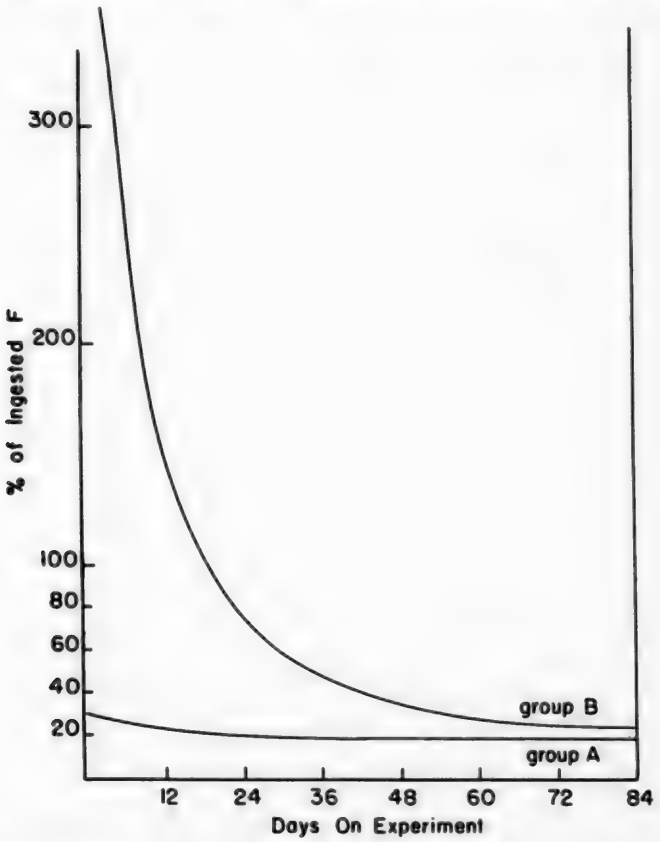

Fig. 2.—Rate of excretion of fluorine in urine by animals which received distilled water during the metabolism study.

Group B animals is extremely high at the beginning of the experiment and decreases rapidly at first, then more slowly as a positive fluorine balance is approached. No difference between these 2 groups in the amount of fluorine excreted in the feces was found. These urine and feces data, showing excretion of large amounts of fluorine in the urine of Group B animals accompanied by

no increase in the amount of fluorine in the feces, seem to indicate that the fluorine found in the feces in these and similar animals was present as a result of nonabsorption, and thus should not be considered available for metabolism.

Fig. 3 shows the amount of fluorine found in the feces, in terms of amount found to amount ingested, by those animals receiving 5 μg F per milliliter. The Group A′ animals showed some difference in the percentage of ingested fluorine recovered from the feces of the males and females. The Group B′ animals did

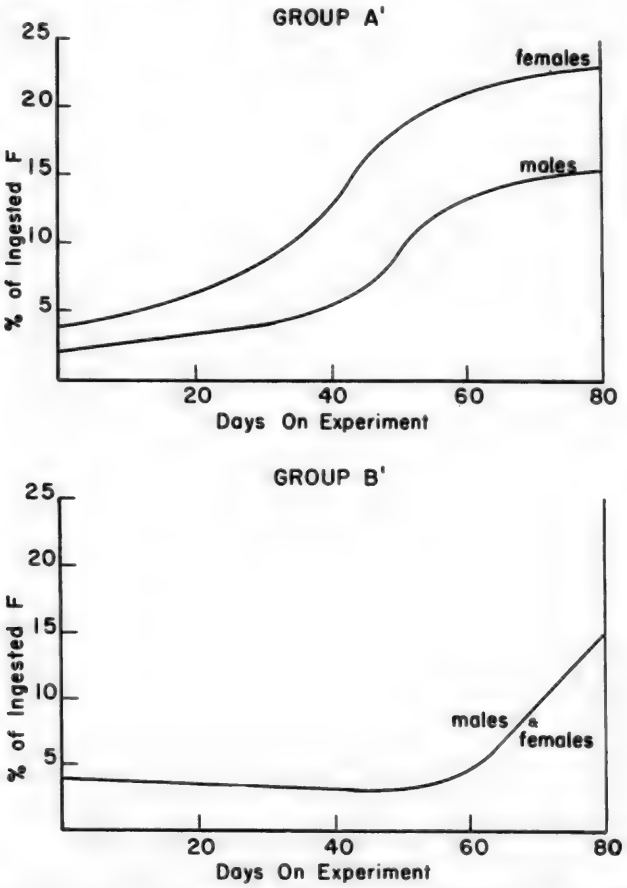

Fig. 3.—Rate of excretion of fluorine in feces by animals drinking 5 μg F per milliliter during the metabolism study.

not show this sex difference. The rate of storage of fluorine by the animals receiving 5 μg F per milliliter in the drinking water was determined, assuming the difference between the amount ingested and the amount excreted to be the amount stored. The rate was calculated as the amount of fluorine retained (in micrograms) per day per 100 grams of body weight. These results are shown in Fig. 4. The Group B′ animals stored fluorine at a higher rate than the corre-

sponding Group A′ animals. However, Group A′ males ingested 142 μg F per day as compared to 164 μg F per day ingested by the Group B′ males; similar data for the females of these groups showed an average of 106 μg F ingested per rat per day by Group A′ females and 150 μg F ingested by Group B′ females. Since the Group B′ animals ingested more fluorine per day, this probably accounts for the somewhat higher storage rate. In all cases, a similarly decreasing rate of storage was observed as the experiment progressed. This was observed regardless of previous contact with the element, indicating that the ingestion of large amounts of fluorine for a short period of time will not greatly affect the subsequent metabolism of more nearly normal amounts such as might

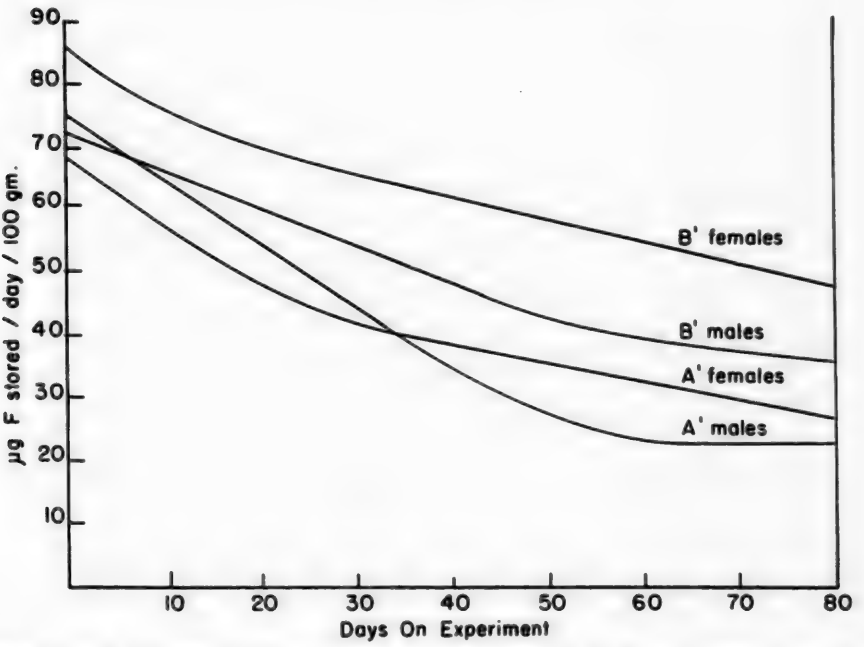

Fig. 4.—Rate of storage of fluorine by animals drinking 5 μg F per milliliter during the metabolism study.

be encountered in fluoridized communal water supplies or from normal dietary sources. The females retained fluorine at a higher rate (expressed in micrograms F per 100 grams of body weight per day) than the corresponding males. This seems to indicate that the females have a greater relative capacity for storage of fluorine than the males. The percentage of ingested fluorine retained during each 3-day period was calculated. These data indicate that the percentage of ingested fluorine retained by Group A′ was greater than the percentage retained by Group B′ animals during the first 15 days. After about 50 days on the experiment, the Group A′ animals stored a smaller percentage of the ingested fluorine than the group B′ animals.

The total amount of fluorine assumed to have been retained during the experiment was calculated and plotted against time. Fig. 5 represents the 2 groups of animals which received 5 μg F per milliliter and Fig. 6 represents the 2 groups of distilled water animals. The Group A′ animals appear to approach a linear retention of fluorine, while the animals of Group B′ seem to store smaller amounts initially, but after about 24 days, they also show a linear

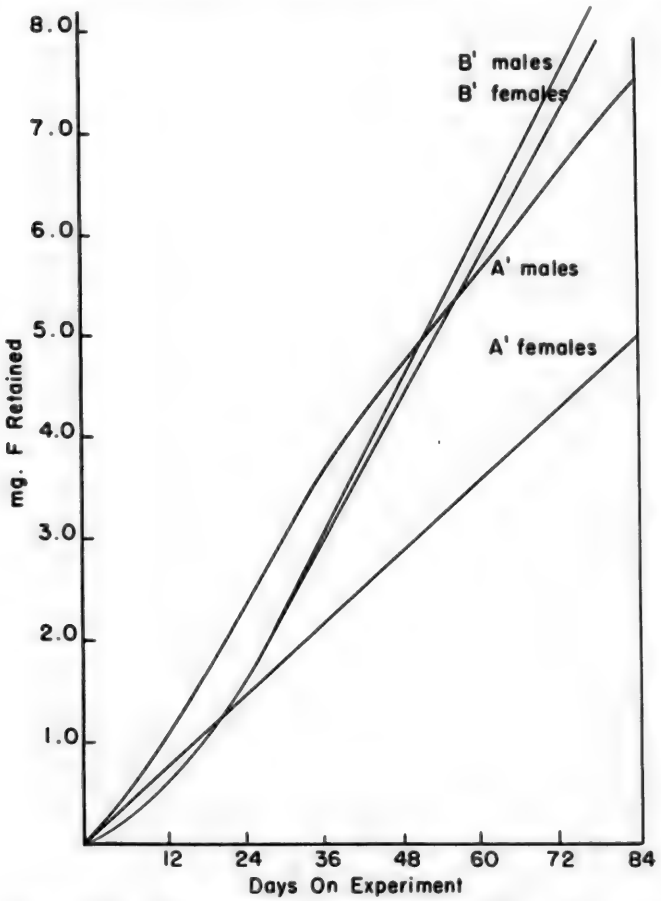

Fig. 5.—Total fluorine retained by animals drinking 5 μg F per milliliter during the metabolism study.

storage. This seems to indicate that their fluorine balance may have been disturbed by the larger amounts of fluorine ingested before the metabolism study began. The Group B′ males showed the greatest total fluorine retention, followed by Group B′ females, then Group A′ males. The Group A′ females showed a much lower fluorine retention. However, having ingested less water and food, this finding is undoubtedly a result of receiving less fluorine.

The data for Fig. 6 indicate that the Group B animals reached a point of minimum total fluorine content after about 30 days on the metabolism study as indicated by the minimum of the curve. Thereafter, the slope indicates positive storage, and after 40 days, the storage rate of the Group B animals was about the same as that of the Group A (control) animals.

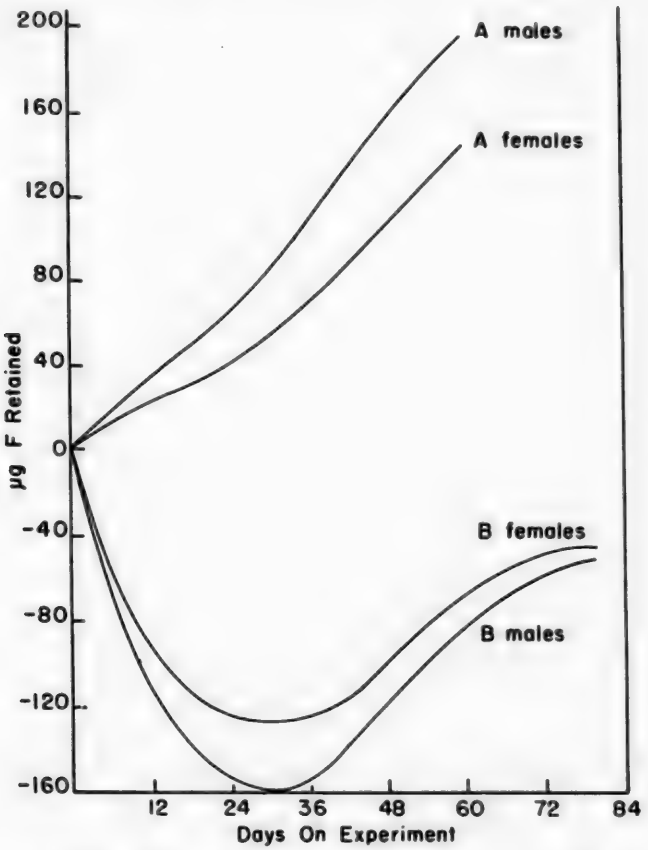

Fig. 6.—Total fluorine retained by animals drinking distilled water during the metabolism study.

At the termination of the metabolism study, the animals were sacrificed, and the femurs removed for fluorine analysis. Table I is a summary of these data. It shows the same relationships between the groups, when the total amount of fluorine in the femur is considered, as was observed from the total amount retained as determined from the metabolism studies. This seems further to indicate that there are probably not any grossly inconsistent errors in this method for studying metabolism of fluorine in the same rat throughout the experiment. The femurs of the animals of Group B which received distilled water contained more fluorine than those of the Group A distilled water animals.

The distribution (in percentage form) of the fluorine ingested was calculated for the various groups and is presented in Table II. These data show that about 35 per cent of the fluorine occurring naturally as a constituent in the diet was retained by the animal. However, approximately 64 per cent of the fluorine administered as a solution of sodium fluoride (5 μg F per milliliter) is retained. This seems to indicate that fluorine in solution is more readily absorbed, and thus more "available" than fluorine occurring naturally in the diet. Also, previous exposure to moderate levels of fluorine does not apparently affect the ultimate storage rate of smaller fluorine concentrations, since the B′ animals also retained about 68 per cent of the ingested fluorine.

TABLE I
FLUORINE CONTENT OF FEMURS

GROUP	SUPPLEMENT WHILE ON EXPERIMENT	SEX	DESSICATED WEIGHT (GM.)	ASHED WEIGHT (GM.)	PER CENT ASH	F. CONC. IN FEMUR (μg F/GM.)	TOTAL FLUORINE IN FEMUR (MG.)
A	Distilled water	M	0.7100	0.4216	59.4	90.6	0.038
		F	0.4571	0.2656	58.1	75.0	0.020
A′	NaF in distilled water (5 μg F/ml.)	M	0.6486	0.3920	60.4	676.6	0.264
		F	0.4344	0.2437	56.1	698.6	0.165
B	Distilled water	M	0.5802	0.3390	58.4	154.1	0.052
		F	0.4688	0.2702	57.6	188.5	0.051
B′	NaF in distilled water (5 μg F/ml.)	M	0.6134	0.3678	60.0	880.5	0.324
		F	0.5134	0.3056	59.5	924.2	0.284

TABLE II
RELATIVE RETENTION AND EXCRETION OF FLUORINE

GROUP	SUPPLEMENT	SEX	INGESTED (MG. F)	FECES (%)	URINE (%)	RETAINED (%)
A	Distilled water	M	0.597	36.2	31.2	32.6
		F	0.443	36.3	26.6	37.1
A′	NaF (5 ppm)	M	11.927	8.9	27.4	63.7
		F	8.756	14.9	29.3	55.8
B	Distilled water	M	0.638	37.4	73.9	−11.3
		F	0.579	40.4	67.5	−7.9
B′	NaF (5 ppm)	M	13.293	5.8	26.7	67.5
		F	12.160	4.9	27.1	68.0

SUMMARY

A method has been devised for the study of the metabolism of fluorine by the rat which allows the individual rat to be used throughout the experiment, thus eliminating, or at least reducing, the problem of biologic variance which is so frequently encountered even when littermates are compared. Using this method, it has been observed that the rate of retention of fluorine is apparently affected only during the first few days following the administration of fairly large doses of the element. However, the effect is not noticeable when the animal is maintained on a moderate concentration of fluorine for a relatively long period of time thereafter. This method may now be further applied to the study of metabolic availability and storage of fluorine in the presence of various inorganic ions. A few of those which are deserving of study are stannous tin (SnF_2 and SnClF), calcium, aluminum, magnesium, iron, and the

anions, monofluorophosphate and fluosilicate. Perhaps one of the most important applications of this method is its utility in measuring the biologic "availability" of different chemical forms of fluorine since the metabolism (absorption, excretion, and storage) is the only sure criterion of availability.

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EFFECT OF DESALIVATION ON ADRENALS, UTERUS, AND TESTES IN THE RAT

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THE effects of chronic and acute inanition on the endocrine glands have been previously reported.[1-3] The characteristic picture of the testes, ovaries, and uterus in acute starvation is one of hypofunction and atrophy. The response of the adrenal gland to limited feedings is somewhat different; Mulinos and Pomerantz[1] and Sarason[4] have reported a slight increase in weight of the rat adrenal, while D'Angelo, Gordon, and Charipper[5] concluded that adrenal enlargement is a variable response to inanition in the rat. D'Angelo, Gordon, and Charipper[6] found an increase in adrenal weight in the guinea pig and concluded that the increase is roughly proportional to the degree of body weight loss and the duration of inanition. That the duration of inanition is important is further supported by the work of Gordon and Katsch,[7] who subjected rats to a restricted diet for 28 days and found that the absolute adrenal weights were unaffected. In the present series of experiments, the effects of inanition were felt to be of importance and, hence, worthy of careful consideration, since the removal of the major portion of the salivary glands, as previous work has indicated,[8] can result in a decreased food intake and, as a result, a partial inanition. Hence, the purpose of this study was to determine the effects of removal of salivary tissue upon the endocrine system, dental caries, and fluorine storage. The data, pertaining to fluorine storage and dental caries, have been reported.[8]

METHODS

In Series I of these studies, 144 weanling rats were equally divided as to sex, initial body weight, and general appearance into 4 different groups, each of which was composed of equal numbers of males and females. One group was gonadectomized, another was desalivated,[8] and a third both gonadectomized and desalivated. The fourth group was unoperated and served as controls. All the animals were maintained on our cariogenic, coarsely ground corn diet[9] and distilled water ad libitum for the experimental period of 140 days, in order that dental caries studies could be conducted on these animals. At the end of the experimental period, the animals were sacrificed by ether inhalation, and the uterus, ovaries, pancreas, thyroid, adrenals, and testes were removed and fixed in 10 per cent Formalin for histologic study.

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The uterus, testes, and adrenals were first blotted dry on filter paper and weighed on an analytic balance, and blood was taken from each animal by the hepatic portal vein and analyzed for calcium[10] and phosphorus.[11]

In Series II, a total of 72 animals were divided equally as to sex into 2 experimental groups. One group was desalivated and the other group was unoperated and maintained as the control. Following the operation, each of these desalivated animals was paired to a control animal of the same sex and initial body weight. The desalivated partner was allowed food ad libitum and his daily consumption measured, while the control animal received as his daily food supply only the amount of food consumed by the operated partner. Distilled water was available in an unlimited supply to both groups.

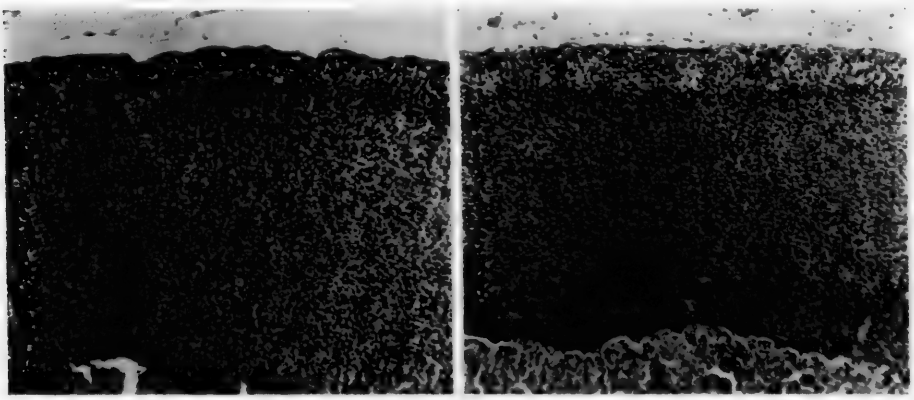

Fig. 1.—Adrenal glands of control male (left) and desalivated male (right). Infiltration of glomerulosa by lipoid-filled cells is noted in the operated animal.

RESULTS AND DISCUSSION

In Series I, no significant differences were noted in the calcium and phosphorus concentrations of the blood serum of the different groups. Histologic examination of the ovaries, thyroid, uterus, and testes did not reveal any significant differences between the desalivated and control groups. However, histologic changes were found in the adrenal glands of the desalivated animals. There was a widening of the zona glomerulosa, which appeared to be principally due to the presence in this layer of large lipoid-filled cells resembling those of the zona fasciculata, but having a much clearer appearance. In addition, there was a condensation or "layering" of cells with large vesicular nuclei but poorly defined cell outlines beneath the glomerulosa (Fig. 1). These alterations were not present in the entire group of desalivated animals to the same degree, nor were the changes as prominent in the female desalivates as in the males. In the gonadectomized group, only an occasional animal showed these changes, but in the gonadectomized-desalivated rats structural alterations comparable to those noted for desalivation were apparent. Here also, these cortical changes were more striking in the males than in the females.

It has been shown[12, 13] that the zona glomerulosa of the adrenal gland is directly concerned with the production of desoxycorticosterone and its control of the body salt-water balance. The gross histologic changes reported here suggest that the removal of salivary tissue may directly affect the salt-water balance in the rat. It is interesting to note that in previous uncontrolled experiments, desalivated animals drank less water per day than unoperated controls.

TABLE I
SUMMARY OF DATA FROM UNRESTRICTED FEEDING EXPERIMENT

GROUP	NO. OF RATS	BODY WT. GAIN (GM.)	MEAN ADRENAL WT. (MG./100 GM.)	MEAN TESTIS OR UTERUS WT. (MG./100 GM.)	ADRENAL t VALUES*
			Males		
Control	18	320	246	15.4 ± 2.4†	865 ± 98	—
Desalivate	16	283	212	15.8 ± 3.0	986 ± 107	0.45
Castrate	14	284	214	19.8 ± 2.4	—	6.0
Castrate and desalivate	15	245	169	18.9 ± 2.7	—	4.5
			Females		
Control	16	212	143	24.5 ± 4.5	180 ± 38	—
Desalivate	14	199	127	30.6 ± 4.1	205 ± 32	3.9
Castrate	13	254	181	23.1 ± 4.2	21 ± 7	0.52
Castrate and desalivate	12	248	177	21.4 ± 2.9	14 ± 4	2.2

*Compared to control.

†Standard deviation $= \sqrt{\frac{\Sigma\,|d|^2}{n-1}}$.

The organ weights of the various groups in Series I are presented in Table I. Orchiectomy resulted in an increased adrenal weight, which is in accord with observations by other investigators.[14, 15] This difference is significant with a probability of less than 0.01. Desalivation in the male had no significant effect upon the adrenal weight, while those animals which were desalivated and castrated showed adrenal weight changes of the same magnitude and significance as the orchiectomized rats. Ovariectomy, on the other hand, had no significant effect upon the adrenal weight. Smith[16] has reported similar results, although other investigators have found that a decreased adrenal weight following ovariectomy is a more consistent response.[14] Desalivation in the female, however, produced a significant increase in adrenal weight (probability < 0.01). In the females that were both castrated and desalivated, there was a significant reduction in adrenal weight. Thus, it appears that the histologic changes do not correspond to the weight changes since the males exhibited the most prominent histologic alterations and the females showed the weight changes. In the Series II animals, the adrenal data (Table II) indicate that the adrenal weight changes may be of a nutritional nature and are dependent, at least in part, upon food intake. These data seem to account for the somewhat inconsistent adrenal weight changes in the operated female groups. The histologic alterations in the desalivated adrenals, however, are suggestive of a dysfunction in the salt-water balance of

the body, since it has been shown that the zona glomerulosa of the cortex is the probable site of origin of the hormone controlling the salt-water balance, desoxycorticosterone.[12, 13]

TABLE II
SUMMARY OF DATA FROM PAIRED FEEDING EXPERIMENT

GROUP	FINAL WEIGHT (GM.)	WEIGHT GAIN (GM.)	FOOD CONSUMPTION PER DAY (GM.)	WATER CONSUMPTION PER DAY (ML.)	ADRENAL WEIGHT (MG./100 GM.)	t VALUE FOR ADRENALS	UTERUS OR TESTIS WT. (MG./100 GM.)	t VALUE FOR UTERUS AND TESTIS
				Males				
Control	343	257	17.6	22	13.3 ± 1.3*	—	907 ± 75	—
Desalivate	315	237	17.8	20	14.6 ± 2.8	0.68	924 ± 68	0.55
				Females				
Control	226	147	14.9	21	25.3 ± 2.7	—	154.7 ± 24	—
Desalivate	210	143	15.0	17	28.0 ± 4.2	1.73	205.0 ± 68	2.30

*Standard deviation.

Ovariectomy produced a marked atrophy of the uterus, while desalivation resulted in an increase in uterine weight, although the significance of this increase is questionable. However, desalivation did produce a significant increase (probability < 0.01) in the testis weight, although no gross histologic differences were apparent. Because of the varied responses obtained here in desalivated animals, and also because it was observed that these animals apparently ate less food than the control animals, it was deemed important to investigate these weight changes under conditions of controlled food intake. A second series of approximately 70 animals were pair-fed under conditions which have already been described. The data of the experiment are presented in Table II.

The adrenal weight changes which occurred in the desalivated females in Series I were markedly reduced in the pair-fed desalivated females. The increase found here was not significant at the 5 per cent level of confidence. It is also very interesting to note that the testis weight changes manifested in Series I were completely lacking in Series II, while the uteri of the desalivated females in Series II were significantly increased in weight. The results from this series of animals indicate very strongly that the basic underlying cause of the adrenal and testes changes in desalivated animals is food intake, and thus nutritional in nature. Unpublished results from this laboratory indicate that desalivated animals eat approximately 1 to 2 Gm. of food a day less than control animals under adlibitum conditions. However, Leathem[17, 18] has found that slight decreases in food consumption which are of the order of 3 Gm. per day do not significantly affect the testis and adrenal weights. Although this points to some specific nutritional need that is lacking in the desalivated animal, it must be remembered that these animals were on a restricted food intake for 140 days. That the duration of inanition is important has already been pointed out by Gordon and Katsch,[7] and this probably accounts for the unusual organ weight changes found here.

SUMMARY

Desalivated male rats showed changes in the adrenal glands consisting of a widening of the zona glomerulosa. This appeared to be principally due to the presence in this layer of large lipoid-filled cells resembling those in the zona fasciculata. The female desalivated rats showed similar but less-pronounced changes. Under ad libitum feeding conditions, desalivation resulted in adrenal weight changes in the females which did not occur in a pair-feeding experiment. The testes were not significantly altered under pair-feeding conditions, although the uteri of desalivated animals significantly increased in size in both cases.

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CHARACTERISTICS OF A PROTEOLYTIC ENZYME IN THE SUBMAXILLARY AND SUBLINGUAL GLANDS OF THE ALBINO RAT

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SALIVARY glands have long been known to produce amylase and mucin. Saliva, on the other hand, has been known to contain mucin and amylase, and also to show some proteolytic activity.[5] Protease activity of the salivary glands had not been demonstrated until Junqueira, Fajer, Rabinovitch, and Frankenthal[2] showed that extracts of the submaxillary glands of mice could degrade casein. A preliminary study showed that the submaxillary gland of the rat also had proteolytic activity. The present investigation is concerned with the characteristics of this enzyme system.

MATERIALS AND METHODS

Twenty-five adult, male rats of the Sprague-Dawley strain were used. The animals were killed by ether, and the capsules containing the submaxillary and sublingual glands were dissected free of the adjacent organs, placed in liquid air, and then transferred to a Gersh-Altmann freeze-drying unit for lyophilization.

An extract was made by grinding the finely cut frozen-dried glands in a mortar and slowly adding saline or buffer solutions. This crude material was then centrifuged for 5 minutes at 1,750 r.p.m. The enzymatic studies were done on the resultant supernatant fluid. Protease activity was determined using either casein or azocoll* as a substrate.

FINDINGS

(A) *Substrate Specificity of Gland Extracts.*—

In addition to azocoll the following substrates and tissue elements were tested for breakdown: casein, gelatin, benzoyl-arginine-amide, collagenous tissue, gastric mucin, and chondroitin sulfate.

1. *Casein:* Casein breakdown was measured by the method of Junqueira, Fajer, Rabinovitch, and Frankenthal.[2] The gland extract liberated twice as much tyrosine as was contained in the standard, indicating fairly rapid breakdown of the casein.

2. *Gelatin:* Liquefaction of gelatin by gland extracts was noted after 24 hours of incubation at pH 7.0.

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*As far as the salivary gland protease is concerned, it was found that azocoll and casein could usually be substituted for one another without affecting the results. Unless otherwise specified, azocoll was the substrate used.

3. *Benzoyl-arginine-amide:* Gland extracts were incubated with benzoyl-arginine-amide for 1 to 3 hours and then tested for liberated ammonia by the method of Folin and Bell.[1] The extract of the salivary glands could degrade benzoyl-arginine-amide nearly as rapidly as trypsin.

4. *Gastric mucin and chondroitin sulfate:* The breakdown of gastric mucin and of chondroitin sulfate was determined by testing for the presence of reducing groups.[1] No evidence of a reducing substance was seen in the solutions. The gland extracts could not degrade either gastric mucin or chondroitin sulfate.

5. *Collagenous tissue:* The tail tendon of the rat was used as the source of collagen. This was dissected out, extracted at 0° C. with ether and acetone, and lyophilized. Identical dry weights of the diced frozen-dried tendon were incubated for successive 24-hour periods with a saline extract of the glands at pH 7.0. The amount of tendon tissue put into solution after each period was determined from the dry weight of the residual undissolved tendon. It was found that gland extracts did not decrease the weight of undissolved tendon compared with saline solution controls.

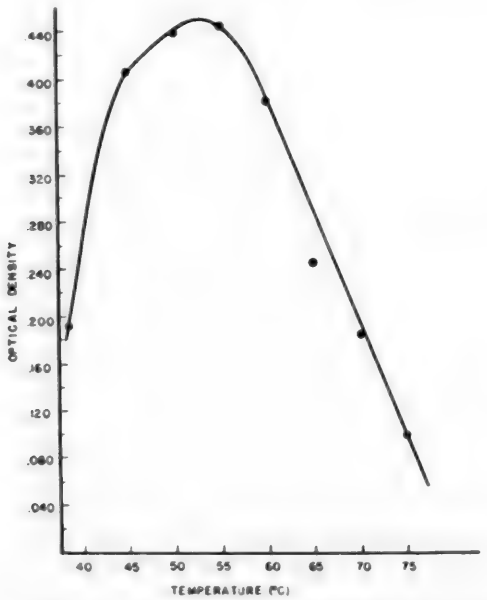

Fig. 1.—Influence of temperature on submaxillary gland protease.

B. *Conditions of Proteolytic Activity.*—

The influence of the following factors was examined: (1) temperature, (2) pH, (3) effects of acid and alkali, (4) inhibitors and activators, (5) effect of trypsin inhibitor, (6) dialysis.

1. *Temperature:* Fourteen-minute periods of enzyme action were used to establish the temperature curve. The effect of temperature on enzyme activity is shown in Fig. 1. At 55° C. there is a well-defined maximum. Enzyme activity is completely abolished by heating to 100° C.

2. *pH:* Phthalate, KH_2PO_4, and boric acid were used as buffers in a final concentration of 0.05 M. There was maximal activity between pH 8.5 and 9.5 (Fig. 2), and rapid dropping off on either side of the maximum. The smoothness of the curve in Fig. 2 indicates that the changes observed are due to changes in hydrogen ion concentration and not to the buffer ions used. The shape of the curve was the same with casein as a substrate.

3. *Effects of acid and alkali:* Gland extracts were exposed to the tested hydrogen ion concentrations for ninety minutes and then tested for enzyme action at the pH maximum (9.6), using casein as a substrate. The enzyme system was mildly affected by exposures to pH's on the acid side of its optimum pH and markedly affected by pH's above this optimum (Fig. 3).

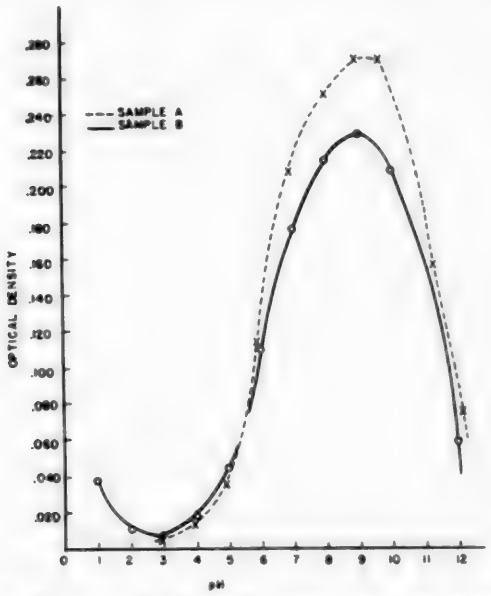

Fig. 2.—Influence of pH on submaxillary gland protease.

4. *Inhibitors and activators:* The following activators and inhibitors were tested in final concentrations as follows:

Cysteine	0.025 Molar
Ascorbic acid	0.025 Molar
Iodine	0.0025 Molar
Magnesium sulfate	0.0005 Molar
Sodium cyanide	0.0025 Molar

Cysteine and ascorbic acid markedly activated the enzyme system; iodine inhibited it, and sodium cyanide and magnesium sulfate had little or no effect upon it.

5. *Effect of trypsin inhibitor:* The effect of Lima bean trypsin inhibitor was tested because of the resemblance of the enzyme system of the salivary glands to

trypsin. The protease of the salivary glands was found not to be inhibited by concentrations of inhibitor which practically abolished the action of equivalent quantities of trypsin.

6. *Dialysis:* Dialysis against distilled water at 0° C. for 48 hours resulted in a 55 per cent reduction in activity. Return of either the original lyophilized dialysate or of an ashed dialysate restored activity to its original value. It was concluded that some inorganic factor is necessary for maximal activity. On the other hand, none of the organic components of the gland extract which can be removed by dialysis are necessary for enzyme action.

C. *Influence of Concentrations of the Enzyme and Its Substrate.*—The findings so far reported should be taken as referring to the submaxillary gland only, because the sublingual gland does not demonstrate any proteolytic activity when azocall is used as a substrate. The findings reported in the present section refer to both glands. It should be noted, however, that the weight of the sublingual gland is about 20 per cent of the combined gland weight.

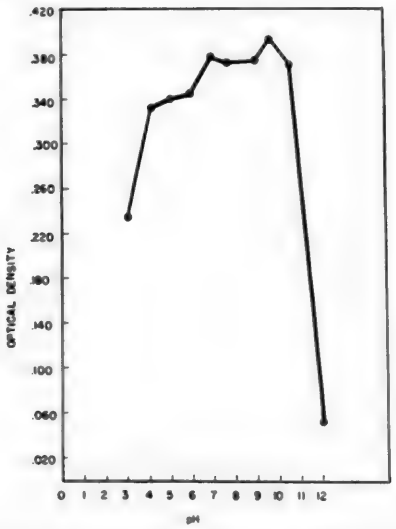

Fig. 3.—Effects of acid and alkali on submaxillary gland protease.

1. *Limiting substrate concentration:* The use of azocoll was not found practical because its concentration could not be raised enough to saturate our customary extract concentrations. With casein, this was very nearly possible, and an approximate determination of its limiting concentration was made, using a pH of 9.6, incubation period of 30 minutes at 37° C., and 5 mg. dry weight of gland tissue. Under these conditions the limiting concentration of casein was about 1.0 per cent.

2. *Limiting velocity of the enzyme:* The limiting velocity of the enzyme was determined from the figures obtained in C 1 according to the method of Lineweaver and Burk.[3] Fig. 4 shows how closely the S/v values followed a

straight line. The limiting velocity (V) equaled 0.48 mg. of tyrosine. The intercept with the ordinate, which in Lineweaver and Burk's equation equals Ks/V, lay at 0.18. The value for K_s was 0.086 per cent casein.

3. *Varying enzyme concentration:* The effect of varying enzyme concentration is shown in Fig. 5. Using the limiting substrate concentration determined in C 1, decreasing enzyme concentrations showed proportionately decreased amounts of tyrosine liberation.

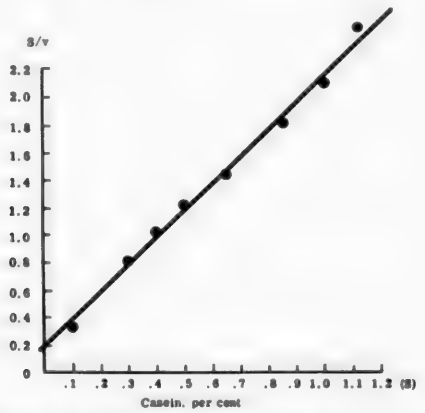

Fig. 4.—Effects of substrate concentration on the reaction velocity.

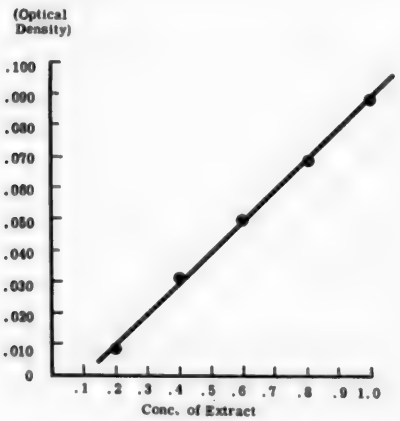

Fig. 5.—Effects of extract concentration on salivary gland protease.

SUMMARY AND CONCLUSIONS

1. The submaxillary and sublingual glands of albino rats contain considerable amounts of an enzyme system which can act on casein, gelatin, and other proteins, but not on mucin or chondroitin sulfate, and therefore appears to be a relatively nonspecific proteolytic system.

2. This enzyme system has its optimal temperature at 55° C., and its optimal pH at between 8.5 and 9.5. It is mildly sensitive to acids and markedly sensitive to alkali.

3. Enzyme activity is increased by cysteine and ascorbic acid, inhibited by iodine and dialysis, and unaffected by trypsin inhibitor, sodium cyanide, and magnesium sulfate.

4. The action of the enzyme system follows the monomolecular reaction curve. The limiting substrate concentration is at about 1.0 per cent casein; the limiting velocity is 0.48 mg. of tyrosine liberation, and the Michaelis constant is 0.086 per cent casein.

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THE RATE OF ENDOGENOUS OXYGEN CONSUMPTION IN BOVINE DENTAL PULP

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INTRODUCTION

UNTIL recently most ideas of the physiologic character of the dental pulp have been drawn inferentially from comparisons of histologic observations on it and other tissues of similar structure. The small total volume of an individual human dental pulp has been a factor that has discouraged physiologic studies of this tissue. The relatively new methods of histochemistry and cytochemistry and quantitative microchemical and microphysiologic techniques appear to be especially applicable to this organ and will undoubtedly become highly important in future studies. However, the use of more traditional methods is not excluded if one chooses experimental animals in which the volume of dental pulp is adequate.

Tissue respiration is a complex of energy-yielding reactions occurring within component cells, and since oxygen consumption is an important aspect of respiration, its measurement affords some insight into the dynamic status of tissue. Manometric techniques for the study of biochemical and physiologic phenomena have become increasingly popular during the past quarter century but their application to dental tissues has been extremely limited. Pincus,[1] in 1950, reported the QO_2 of normal human dental pulp as 0.31.

The present investigation utilized the direct method of Warburg. It was undertaken to secure additional data on endogenous oxygen consumption in dental pulp. The selection of bovine material was influenced largely by its availability and by the large size of individual pulps. Large pulp volume satisfies the requirements of the Warburg apparatus without need for tissue pooling. A secondary factor in its selection was the opportunity it afforded for comparative physiologic observations.

EXPERIMENTAL METHOD

First and second maxillary molars of 1- to 2-year-old cattle were obtained at a local abattoir immediately after killing the animal. The teeth were split and the pulp extracted and immediately placed in chilled Krebs-Ringer's[2] solution. The pulps were cleaned and then sliced with a Stadie-Riggs slicer. Only the central portions of the pulps were used for the experimental determinations.

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All tissue slices selected were less than 1 mm. thick, the maximum permissible thickness being 3.5 mm., according to Warburg's formula.[3] Samples ranging from 0.2 gram to 1.1 grams wet weight were transferred to 5 ml. Warburg reaction flasks within 2 hours after the death of the animals.

Each flask contained 1 ml. of oxygenated Krebs-Ringer–phosphate solution adjusted to pH 7.4 as the suspending medium.[2] The center well of each flask contained 0.5 ml. of 10 per cent KOH solution for CO_2 absorption. Oxygen constituted the gas phase in the flask-manometer system. Manometers with ¼ sq. mm. bore were used. The water bath of the Warburg apparatus was set at 38° C. and the shaking mechanism was adjusted to 115 cycles per minute.

After manometric observations were completed, samples were removed from the flasks, blotted, and wet weight determined. Drying in an oven at 106° C. for a minimum of 6 hours preceded dry weight determinations.

In these experiments the wet weights of tissue samples (assuming a density of 1.0) were subtracted from the total gas volume of flasks and manometers and the resultant values were utilized to determine flask constants.

RESULTS

The results reported in Table I include only those obtained after all procedures had been standardized in actual performance and the experiments had become consistent. The QO_2 expresses the number of microliters of O_2 consumed per milligram of dried tissue per hour.

Oxygen consumption observed during the first hour of the experiment is regarded as the most typical because of degenerative changes that occur in tissue after removal from its normal environment. The rise noted in succeeding hours probably is caused by bacterial proliferation.

TABLE I
OXYGEN UPTAKE OF BOVINE DENTAL PULP

TIME (MIN.)	NUMBER OF ANIMALS	NUMBER OF DETERMINATION	QO_2 (μL/MG./HR.)	STANDARD DEVIATION	RANGE
60	7	54	0.56	0.13	0.26-0.90
120	7	48	0.58	0.14	0.30-0.90
180	7	33	0.71	0.17	0.32-0.96
240	3	23	1.21	0.48	0.63-2.52

DISCUSSION

The observed rate of endogenous oxygen consumption in pulps of fully or almost fully developed teeth is higher for bovine tissue than that reported for human material. Both studies indicate that it is lower in dental pulp than in most other body tissues reported.[4] The observed difference between human and bovine pulp is small when compared to other tissues but when considered from the viewpoint of low respiratory level the difference seems more significant. The difference might be due to experimental factors. However, a previous study of nitrogen content of bovine and human dental pulps indicates a higher concentration in bovine pulp.[5] These combined observations suggest a possible species difference.

It would be unwise to assume that the rate of endogenous oxygen consumption as observed in essentially resting pulps is characteristic of the same tissue active in dentin formation or in its reaction to injury. It is well known that tissue growth and increased functional activity is usually associated with acceleration of respiratory rate.[6] With specific reference to oral tissues it has been shown that oxygen consumption during gingival healing is above that occurring in normal gingiva.[7] The histologic peculiarities of pulps of developing teeth and of those showing chronic inflammatory changes, consequently, leads one to suspect a higher level of metabolism under these circumstances.

Pincus[1] observed that odontoblasts generally remained attached to the dentin when pulps were removed from teeth. We have confirmed this histologically for both human and bovine material. Both studies, therefore, have reported the QO_2 of dental pulp devoid of its odontoblastic layer.

Comparison of the rates of endogenous oxygen consumption provides a means of relating the metabolic status of various tissues. While this knowledge is of value in itself, its usefulness does not end there. "During investigations of the metabolism of exogenous substrates, knowledge of the rate of endogenous metabolism is important because quantitative manometric results are very valuable and in many cases indispensable for understanding metabolic pathways."[8]

CONCLUSIONS

1. The endogenous oxygen consumption in bovine dental pulp has been determined to be 0.56 (S.D. ± 0.13) μl per hour per milligram of dry tissue weight.

2. The higher rate than that reported for human dental pulp suggests a possible species difference and warrants further investigation.

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PRODUCTION OF HYALURONIDASE AND BETA-GLUCURONIDASE BY VIRIDANS STREPTOCOCCI ISOLATED FROM GINGIVAL CREVICES

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IN MARGINAL gingivitis, the intercellular substance disappears from the epithelium lining of the gingival crevice and channels open into the amorphous ground substance of the subjacent connective tissue. This formation of "clefts" in the crevicular epithelium was described first by Stillman.[1]

This phenomenon has been reproduced, with subsequent bacterial invasion of the tissues, by the topical application to clinically healthy gingivae of testicular extracts (hyaluronidase) owing presumably to the depolymerization of the mucopolysaccharides of the gingival tissues by various mucopolysaccharoses.[2] Hyaluronidase and beta-glucuronidase are two of several enzymes present in testicular extracts.[3] Hyaluronidase catalyzes the hydrolysis of glucosaminidic linkages of a mucopolysaccharide tissue-cementing substance, hyaluronic acid, to give a mixture of tetra-, hexa-, and octasaccharides, which are composed of alternating N-acetylglucosamine and glucuronic acid residues. Beta-glucuronidase hydrolyzes off the terminal glucuronic acid residues of these oligosaccharides.[4]

It has been shown that hyaluronidase and beta-glucuronidase are present in subgingival accumulations.[5, 6] It was, therefore, relevant to determine whether or not these enzymes, and others capable of catalyzing the breakdown of elements of the gingival tissue, were produced by microorganisms resident in the gingival crevice. Lisanti has reported the presence of hyaluronidase in saliva and its production by salivary streptococci.[7, 8] Viridans streptococci probably constitute a large portion of the microorganisms that inhabit the gingival crevice.[9] They were, therefore, selected as the first group to be explored in the present investigation.

MATERIALS AND METHODS

Subjects.—Thirty-one normal adults served as sources of cultures. They were selected only for the presence or absence of clinically manifest marginal gingivitis.

Broth Medium.—The broth used throughout this investigation consisted of brain heart infusion broth (Difco), to which was added aseptically, just prior to use, 5 volumes per cent of a solution containing glucose, 4.0 Gm.;

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sodium chloride, 2.0 Gm.; sodium bicarbonate, 2.0 Gm.; disodium phosphate (duodecahydrate), 2.0 Gm.; and distilled water, 50 ml. This solution was sterilized by filtration.

Viridans Streptococci.—In all, 121 single colony strains of viridans streptococci were studied from 17 subjects, plus 40 pools of single colony strains from 20 subjects, 6 of whom served also as members of the first group of 17. In order to minimize salivary contamination, samples were taken only from the buccal gingival crevices of the lower molars or premolars. The subgingival accumulation from one crevice was collected with a gingival scraper and cultivated aerobically at 37° C. for 24 hours on sheep blood agar. Sheep blood was preferred to other bloods because of its greater susceptibility to greening by viridans streptococci. For the single colony strains, at least 6 colonies of viridans streptococci were picked at random from the cultures from each subject, and subcultured on sheep blood agar. A second subculture of each strain on mitis-salivarius agar and sucrose agar was identified as *Streptococcus mitis* or *Streptococcus salivarius* by conventional criteria. Simultaneously, a second subculture was made in broth and checked for the presence of gram-positive cocci in chains. After the addition of sterile skim milk, this culture was stored in the frozen state to provide a supply of stable cultures for subsequent study.

Since preliminary results indicated that not more than 1 single colony strain out of 4 would produce beta-glucuronidase, it seemed probable that a more critical survey would be obtained by the use of pooled strains. For each pool, from 10 to 20 single colony strains each of *Streptococcus mitis* and *Streptococcus salivarius* from a single subject were inoculated into a single tube of broth and the resultant growth was preserved as described previously.

Hyaluronidase.—Each frozen culture was thawed and cultivated on sheep blood agar for 24 hours at 37° C. A sample of the resultant growth was inoculated into broth, which was incubated for 24 hours at 37° C. Because repreentative cultures gave readings of 250 ± 30 in a Klett-Summerson colorimeter with blue filter, standardization was not carried out routinely. Since the hyaluronidase was exocellular, the cells were spun down by centrifugation, and the tests were carried out with aliquots of the supernatant. Activity was determined by the turbidity reduction method outlined by Tolksdorf, McCready, McCullagh, and Schwenk,[10] using hyaluronic acid prepared from human umbilical cords by the tryptic digestion method of Byers, Tytell, and Logan.[11]

Beta-glucuronidase.—The organisms were cultivated as described previously. Since the beta-glucuronidase was endocellular, its activity had to be determined in a suspension of the cells themselves. In order to provide a basis for the comparison of the capability of the various strains to produce beta-glucuronidase, the cellular suspensions were made up to a standard turbidity. To do this, the density of growth in each broth culture was determined in a Klett-Summerson photoelectric colorimeter, using a red filter. A sample of the culture, equivalent to 10 ml. of a suspension giving a colorimeter reading of 250, was then centrifuged and the sedimented bacteria were resuspended in 1.0 ml. of distilled water. The determination was carried

out as described by Jacox,[12] using 0.5 ml. of this suspension, 4.0 ml. of 0.1M phosphate buffer, pH 6.5, and 0.5 ml. of 0.005M sodium phenolphthalein glucuronate (Sigma). The addition of chloroform to the reaction mixture was found to be important, presumably owing to acceleration of the release of the enzyme from the cells. Without it, the hydrolysis of the substrate was delayed for many hours.

RESULTS

Hyaluronidase.—In a preliminary experiment, intended to amplify previous results[5] and to check on the clinical judgment in the choice of subjects, hyaluronidase was produced in 11 cultures from the subjects with gingivitis, but in only 1 of 4 taken from noninflamed gingivae. Statistical analysis showed that even in this small series the difference was most unlikely to have occurred by the chance of fortunate sampling.

TABLE I

PRODUCTION OF HYALURONIDASE BY VIRIDANS STREPTOCOCCI ISOLATED FROM GINGIVAL CREVICES

GROUP	SUBJECTS	STRAINS
Single colony strains		
Total	17	121
Positive/total, gingivitis group	10/10 (100%)	33/71 (47%)
Positive/total, nongingivitis group	2/7 (29%)	3/50 (6%)
Critical ratio	3.1	4.8
Pooled strains		
Total	14	28
Positive/total, gingivitis group	6/6 (100%)	10/13 (77%)
Positive/total, nongingivitis group	2/8 (25%)	3/15 (20%)
Critical ratio	2.8	3.0

This trend was continued in the tests with the pure cultures of viridans streptococci (Table I). Each of the gingivitis subjects gave one or more strains that produced hyaluronidase, whereas only a fourth of the nongingivitis subjects did so. The difference in the incidence of positive single colony strains was even more impressive. All differences were statistically acceptable. As anticipated, the proportion of positive pooled strains was somewhat higher than the proportion of positive single colony strains in both groups of subjects. Since there was no evident difference between the single colony strains of *Streptococcus mitis* and *Streptococcus salivarius*, they have not been segregated in the final tabulation. Hyaluronidase was produced by 26 of the 56 strains of mitis and by 7 of the 15 strains of salivarius isolated from the gingivitis group; and by 3 of the 43 strains of mitis but by none of the 7 strains of salivarius isolated from the nongingivitis group. Beta-glucuronidase was produced by 12 of the mitis strains and 4 of the salivarius strains from the gingivitis group.

The positive strains varied considerably in the amount of hyaluronidase produced. One turbidity reduction unit (TRU) of the enzyme is defined as the amount which, on incubation for 30 minutes at 37° C., reduces the turbidity produced by 0.2 mg. of hyaluronic acid to that produced by 0.1 mg., when added to a standard amount of acidified stabilized human plasma protein. In our series, only strains that produced two TRU or more per milliliter of broth culture were recorded as positive. Most strains were tested twice. Eight strains

produced more than 8 TRU per milliliter; 19 strains, from 6 to 8 TRU per milliliter; 12 strains, from 4 to 6 TRU per milliliter; and 10 strains, from 2 to 4 TRU per milliliter.

Beta-glucuronidase.—As was the case for hyaluronidase, a considerably higher proportion of the gingivitis subjects yielded one or more strains of viridans streptococci that produced beta-glucuronidase, and the over-all proportion of positive strains was higher from this group than from the nongingivitis group (Table II). The statistical analysis was again satisfactory.

TABLE II

PRODUCTION OF BETA-GLUCURONIDASE BY VIRIDANS STREPTOCOCCI ISOLATED FROM GINGIVAL CREVICES

GROUP	SUBJECTS	STRAINS
Single colony strains*		
Total	17	121
Positive/total, gingivitis group	6/10 (60%)	16/71 (23%)
Positive/total, nongingivitis group	0/7 (0%)	0/50 (0%)
Critical ratio	2.6	3.7
Pooled strains†		
Total	20	40
Positive/total, gingivitis group	7/9 (78%)	12/19 (63%)
Positive/total, nongingivitis group	2/11 (18%)	2/21 (10%)
Critical ratio	2.7	3.5

*The same strains as those tested for hyaluronidase.
†Twenty-eight of these strains were tested also for hyaluronidase

Every strain that produced beta-glucuronidase produced hyaluronidase also, but the reverse was not true. Not all gingivitis subjects yielded a glucuronidase-positive strain and the fraction of strains positive was lower. Again, the use of the pooled strains increased the incidence of positive results. In this series, we added 12 pooled strains from 6 of the subjects who had served as sources of single colony strains. In all cases, the results confirmed those obtained in the tests of the single colony strains. All positive strains and many negative strains were tested twice.

The positive strains varied widely in the amount of beta-glucuronidase produced. In each test, the total available phenolphthalein was 795 μg. The lowest value accepted as positive was the liberation of 10 μg of phenolphthalein during incubation at 37° C. for 18 hours. Seven strains liberated from 10 to 30 μg and 15 strains liberated from 30 to 150 μg. The remaining 8 strains hydrolyzed 50 per cent or more of the substrate, but the exact amount was not determined.

Tests for Adaptation.—In general, bacteria produce enzymes either constitutively or adaptively. In the former case, they synthesize the enzyme regardless of the presence or absence of the specific substrate, that is, even when they have no use for it. The tests discussed up to this point were all made with cultures deliberately grown in the absence of the specific substrate. The enzymes were, therefore, produced constitutively. On the other hand, many bacterial enzymes are synthesized adaptively, that is, only when the organism is cultivated in the presence of the substrate or when the cells are incubated with the substrate under such conditions that growth does not occur. In the present instance, one or both of these conditions prevail in the

natural habitat of the organisms under study. It was, therefore, most relevant to determine whether or not the negative strains could produce the enzymes adaptively. If they did so, the significance of our results would be greatly altered. Accordingly, 10 strains that had produced neither enzyme were cultivated in broth containing from 0.04 to 1.0 mg. of hyaluronic acid per milliliter, in all cases for several passages and in one case for 7 passages. In no case was hyaluronidase produced. Similarly, 25 strains that produced neither enzyme were cultivated in broth containing variously menthol-glucuronide, glucuronolactone, acetyl glucosamine, or hyaluronic acid for from 1 to 8 passages. In no case was beta-glucuronidase produced. It was concluded, therefore, that these streptococci do not produce these 2 enzymes adaptively. Furthermore, the failure of the enzymes to appear after serial passages of the cultures in the presence of the substrates shows that the incidence of enzyme-producing mutants is exceedingly low. On the other hand, some of the positive strains produced more of the enzymes under the conditions cited. The 4 strains tested more than doubled their output of hyaluronidase during their first passage in the presence of hyaluronic acid. Seven glucuronidase-positive strains were grown for 24 hours in broth containing 0.04 mg. of menthol glucuronide per milliliter. Three strains were unaffected. Two strains now hydrolyzed twice as much phenolphthalein glucuronide under the standard conditions; one strain, 6 times as much; and one strain, more than 10 times as much. In each case, the comparison was made with a culture of the same strain grown simultaneously in plain broth.

DISCUSSION

The gross similarity of the microbial population of the gingival crevice in gingivitis to that observed normally in the absence of clinically manifest inflammation has rendered it unlikely that any one microorganism is the specific microbial component in this disease. It has also given rise to the view that the increase of the crevicular microorganisms that accompanies the development of gingivitis is a result of the pathologic change and not a cause. The present results, however, show that in gingivitis there can be a significant increase in the incidence of a relevant type of organism, namely, viridans streptococci that produce hyaluronidase and beta-glucuronidase. Previous work[2, 5] indicates that these enzymes contribute to the early stages of marginal gingivitis by opening channels through the crevicular epithelium into the subjacent connective tissue. The occurrence in the nongingivitis subjects of a minor proportion of organisms producing these enzymes does not weaken this working hypothesis. First, it is probable that clinically indetectable foci of reaction were present in the clinically normal gingivae. Second, there may well be factors of host resistance that inhibit either the production of these enzymes or their activity in vivo. The blood serum, at least, frequently contains an antihyaluronidase.

Some of the observed properties of these enzymes may be significant in pathogenesis. The extracellular occurrence of the streptococcal hyaluronidase means that in the natural habitat it would presumably be available to attack tissue at a distance from the actively growing organism. In contrast, the

beta-glucuronidase remained associated with the cell during culture and became active only under conditions favoring increased permeability of the bacterial cell, that is, on death or autolysis of the cell. Perhaps most significant of all is the fact that these enzymes were not produced adaptively. In other words, the ability to synthesize these enzymes is not an attribute of every oral viridans streptococcus, provided that it is cultivated in the presence of the specific substrate, as it is in its natural habitat. Rather, only a restricted proportion of these organisms have this ability and they produce the enzymes regardless of the presence or absence of the substrates. It is inferred tentatively that a more specific bacterial component is involved in gingivitis than has been generally assumed.

Finally, it is important to mention that viridans streptococci are not the only gingival bacteria that produce hyaluronidase and beta-glucuronidase. In the course of the present investigation, staphylococcal strains capable of producing both enzymes and diphtheroid strains capable of producing hyaluronidase have been isolated from the gingival crevice.

SUMMARY

Strains of *Streptococcus mitis* and *Streptococcus salivarius* isolated from the gingival crevices of 31 adults, selected only for the presence or absence of clinically manifest marginal gingivitis, were tested for the production of hyaluronidase and beta-glucuronidase. These enzymes are believed on the basis of previous work to contribute to the development of marginal gingivitis. Both the proportion of subjects yielding one or more positive strains and the over-all proportion of strains producing these enzymes were significantly higher in the group having gingivitis. The hyaluronidase was extracellular, whereas the beta-glucuronidase was endocellular. Neither enzyme was formed adaptively, and positive mutants did not arise spontaneously from negative strains. These observations indicate the participation in gingivitis of a more specific group of bacteria than has been generally recognized.

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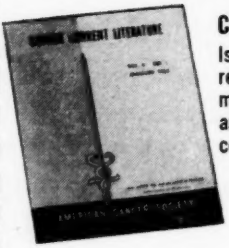

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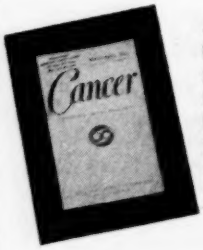

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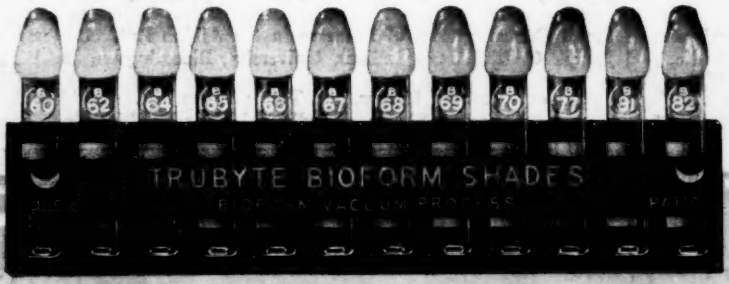

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